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(54) Title: SINGLE-DOMAIN BRAIN-TARGETING ANTIBODY FRAGMENTS DERIVED FROM LLAMA ANTIBODIES

(57) Abstract: A phage-displayed library of llama single heavy domain antibodies (sdAbs) was enriched for species that selectively bind to and are internalized by human cerebromicrovascular endothelial cells (HCEC). From the enriched library, two sdAbs were selected, sequenced, subcloned, and expressed as fusion proteins with c-myc-His₃ tags. Similarly as phage-displayed sdAbs, these soluble tagged sdAbs were shown to selectively bind to HCEC and to transigrate across *in vitro* human blood-brain barrier (BBB) model. In contrast to an unrelated llama sdAb, these sdAbs were also detected in the brain after i.v. injection into mice. These small (~13 kDa) antibody fragments have essential characteristics of brain-specific delivery vectors and can be used to facilitate drug transport across the BBB.

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SINGLE-DOMAIN BRAIN-TARGETING ANTIBODY FRAGMENTS DERIVED FROM LLAMA ANTIBODIES

FIELD OF THE INVENTION

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The invention relates to single-domain brain-targeting antibody fragments derived from llama antibodies, in particular to antibody fragments comprising at least a part of the variable heavy domain (VH or V_HH) of llama antibodies, which fragments selectively bind to and are internalized by human
10 cerebromicrovascular endothelial cells (HCEC), and transigrate HCEC monolayer.

BACKGROUND OF THE INVENTION

15 The immune system in vertebrates provides a defense mechanism against foreign intruders, such as foreign macromolecules or infecting microorganisms. The foreign invaders (antigens), both macromolecules (proteins, polysaccharides, or nucleic acids) and microbes (viruses or bacteria), are recognized through specific binding of the proteins of the host
20 immune system to specific sites on the antigen surface, known as antigenic determinants.

As part of the immune system, B-cells of vertebrate organisms synthesize antigen-recognizing proteins known as antibodies or immunoglobulins (Ig).
25 According to the clonal selection theory, an antigen activates those B-cells of the host organism that have on their surface immunoglobulins that can recognize and bind the antigen. The binding triggers production of a clone of identical B-cells that secrete soluble antigen-binding immunoglobulins into the bloodstream. Antibodies secreted by B-cells bind to foreign material (antigen)
30 to serve as tags or identifiers for such material. Antibody-tagged antigens are then recognized and disposed of by macrophages and other effector cells of the immune system or are directly lysed by a set of nonspecific serum proteins collectively called complement. In this way a small amount of antigen

can elicit an amplified and specific immune response that helps to clear the host organism of the source of antigen. Through a complex process of gene splicing combined with additional mutation mechanisms, human B-cells have been estimated to produce a "library" (repertoire) of more than a billion (10^9)
5 different antibodies that differ in the composition of their binding sites.

For most vertebrate organisms, including humans and murine species, their antibodies show a common structural pattern which consists of two identical light polypeptide chains and two identical heavy polypeptide chains linked
10 together by disulfide bonds and numerous non-covalent interactions, resulting in a Y-shaped molecule. In humans, there are two different classes (isotypes), λ and κ , of the light chains, with no known functional distinction between them. The heavy chains have five different isotypes that divide immunoglobulins into five different functional classes (IgG, IgM, IgA, IgD,
15 IgE), each with different effector properties in the elimination of antigen.

Of the above five classes, immunoglobulins of the IgG class are the major type in normal serum of humans and many other species and have the four-chain structure shown schematically in Fig. 1. Each chain of an IgG molecule
20 is divided into domains of about 110 amino acid residues, with the light chains having two such domains and the heavy chains having four. Comparison of amino acid sequences between different IgGs shows that the amino-terminal domain of each chain (both light and heavy) is highly variable, whereas the remaining domains have substantially constant sequences. In other words,
25 the light (L) chains of an IgG molecule are built up from one amino-terminal variable domain (VL) and one carboxy-terminal constant domain (CL), and the heavy (H) chains from one amino-terminal variable domain (VH) followed by three constant domains (CH1, CH2, and CH3).

30 The variable domains are not uniformly variable throughout their length. Three small regions of a variable domain, known as hypervariable regions (loops) or complementarity determining regions (CDR1, CDR2, and CDR3) show much more variability than the rest of the domain. These regions, which

vary in size and sequence among various immunoglobulins, determine the specificity of the antigen-antibody interaction. The specificity of an antibody of the type shown in Fig. 1 is determined by the sequence and size of six hypervariable loops (regions), three in the VL domain and three in the VH domain.

By partial digestion with papain, which cleaves the heavy chains in the hinge region, the IgG molecule can be broken down into two identical Fab fragments (Fragment, antigen binding) and one Fc fragment (Fragment, crystallizes easily). Each Fab fragment comprises one complete light chain (consisting of VL and CL domains) linked by a disulfide bridge and noncovalent interactions to a fragment of the heavy chain consisting of VH and CH1 domains. The Fc fragment comprises CH2 and CH3 domains from both heavy chains, also linked by disulfide bridges and noncovalent interactions. The part of the Fab fragment consisting of variable domains of the light and the heavy chain (VL and VH) is known as Fv fragment (Fragment, variable). In an Fv fragment, the variable domains VL and VH are not covalently bound. In an scFv (single chain Fv) fragment, the VL and VH domains are covalently linked by a short peptide linker (spacer), usually 15 to 20 amino acids long, introduced at the genetic level (see Fig. 2).

scFv fragments are recombinant fusion proteins and are produced by techniques of genetic engineering, by expressing in a suitable host, usually in bacteria, a chimeric gene coding for the fragment. Various other recombinant antibody fragments have been designed to substitute for large intact immunoglobulin molecules (see Fig. 2). Other than scFv fragments, these options include Fab or Fv fragments that are stabilized or covalently linked using various strategies (see, for example, Bird et al., *Science*, **242**, 423-426 (1988); Huston et al., *Proc. Natl. Acad. Sci. USA*, **85**, 5879-5883 (1988); Glockshuber et al., *Biochemistry*, **29**, 1362-1376 (1990); Jung et al., *Proteins*, 35-47 (1994); Reiter et al., *Biochemistry*, 5451-5459, 18327-18331 (1994); Young et al., *FEBS Lett.*, 135-139 (1995)). Small antigen-binding fragments of

natural antibodies are advantageous for medical applications, for example cancer targeting and imaging, when small antigen-binding molecules are required to penetrate into solid tumors.

- 5 Recent advances in gene technology have greatly facilitated the genetic manipulation, production, identification and conjugation of recombinant antibody fragments and broadened the potential utility of antibodies as diagnostic and therapeutic agents. Of particular importance to such applications is the possibility to alter the fine specificity of the antibody binding
- 10 site, to create small stable antigen-binding fragments, to prepare fusion proteins combining antigen-binding domains with proteins having desired therapeutic properties, for the purpose of immunotargeting, or to "humanize" antibodies of other species, for example murine antibodies (see Fig. 2).
- 15 The genetic engineering has also made possible to screen in vitro for antibodies having a predetermined binding specificity. This may be achieved by constructing first a gene library of antibodies or antibody fragments, for example by polymerase chain reaction (PCR)-amplification of cDNA derived from B-lymphocytes using suitable primers, or by in vitro gene synthesis. The
- 20 gene library may contain sequences corresponding to certain fragments of natural antibodies, or randomized antigen-binding regions, or new combinations of heavy/light chains, thus creating the potential for generating antibodies which could never be obtained from natural sources, for example, antibodies to highly toxic substances or antigens tolerated by the human
- 25 immune system. By random or designed mutations, the affinity or specificity of the antigen binding can be manipulated, for example, to reach affinities never observed with natural antibodies.

To screen a gene library, which may contain many millions or even billions of

30 different clones, for genes of antibodies having the desired binding specificity, a selection system comparable to that of the immune system is required. Such a selection system can be achieved by inserting the library genes into the genome of microorganisms capable of displaying on their surface the

antibody corresponding to the inserted gene, in analogy to the expression of an immunoglobulin antigen receptor on the surface of a B-cell. Microorganisms most frequently used for providing such a display are filamentous bacteriophages, such as fd or M13 phages (phage display). The collection of phage particles having inserted genes of a library of proteins, such as antibodies, and displaying these proteins on the particles' surface is known as a phage display library. The display of the library of antibodies on the surface of phage particles provides a physical link between the antigen-binding function of an antibody and the antibody gene. Using the affinity to a preselected antigen, the whole organism (phage) displaying this affinity can be identified and separated out of billions of non-specific clones, usually through binding to the antigen immobilized on a support, technique usually referred to as panning (see, for example, Scott et al., *Science*, **249**, 386-390 (1990); Winter et al., *Annual Rev. Immunology*, **12**, 433-455 (1994)). Phage clones binding to the antigen can be then amplified and used to produce the specific antibody or antibody fragment in *E. coli* or in other suitable organism.

For naturally occurring antibodies, there are examples that whole heavy chains alone retain a significant binding ability in the absence of light chains. It is also well established, from structural studies, that the CDR3 of the heavy variable domain generally contributes the most to antigen binding, because CDR3 amino acid residues are responsible for most of the surface contact area and molecular interaction with the antigen (Padlan, E.A., *Mol. Immunology*, **31**, 169-217 (1984); Chothia et al., *J. Mol. Biol.*, **196**, 904-917 (1987); Chothia et al., *J. Mol. Biol.*, **186**, 651-663 (1985)). Less binding activity was observed for light chain. In view of these findings, attempts were made to isolate single VH domains. For example, VH domains were isolated from expression libraries derived from immunized mice (Ward et al., *Nature*, **341**, 544-546 (1989)). In another report, antigen-binding VH domains were rescued from an antibody phage library that was made from a vaccinated patient (Cai et al., *Proc Natl. Acad. Sci. USA*, **93**, 6280-6285 (1996)). Antigen-binding antibody fragments consisting of a single VH domain, known

as dAbs or sdAbs (single-domain antibodies), are becoming an attractive alternative to single chain Fv (scFv) fragments. Despite smaller binding surface, their demonstrated affinity is comparable to that demonstrated by scFv fragments (Davies et al., *Biotech.*, **13**, 475-479 (1995)). Because of their smaller size, being half of the size of scFvs, sdAbs are amenable to detailed NMR structural studies (Davies et al., *FEBS Letters*, **339**, 285-290 (1994)). Additionally, due to their simpler structure, sdAbs are more stable and have simpler folding properties.

10 Recently, a new class of antibodies known as heavy chain antibodies (HCA, also referred to as two-chain or two-chain heavy chain antibodies) have been reported in camelids (Hamers-Casterman et al., *Nature*, **363**, 446-448 (1993); see also US 5,759,808; US 5,800,988; US 5,840,526; and US 5,874,541). Compared with conventional four-chain immunoglobulins of IgG-type, which are also produced by camelids, these antibodies lack the light chains and CH1 domains of conventional immunoglobulins. One of the salient features of these naturally occurring heavy chain antibodies is the predominant presence of Glu, Arg and Gly at VL interface positions 44, 45 and 47 (Kabat numbering), respectively, of their variable domain (designated V_HH). The same positions in the variable domain of the heavy chain of conventional four-chain antibodies (designated VH) are almost exclusively occupied by Gly, Leu and Trp. These differences are thought to be responsible for the high solubility and stability of camelid HCA variable domain (V_HH), as compared with the relative insolubility of VH domain of the conventional four-chain antibodies. Two more salient features of camelid V_HH domains are their comparatively longer CDR3 and high incidence of cysteine pairs in CDRs. It appears that cysteine pairs mediate the formation of a disulfide bridge and are therefore involved in modulating the surface topology of the antibody combining site. In the crystal structure of a camel sdAb-lysozyme complex, a rigid loop protruding from the sdAb and partly stabilized by a CDR disulfide linkage extends out of the combining site and penetrates deeply into the lysozyme active site (Desmyter et al., *Nature Struct. Biol.*, **3**, 803-811 (1996)).

More recently, a number of camelid sdAbs phage display libraries have been generated from the V_HH repertoire of camelids immunized with various antigens (Arbabi et al., *FEBS Letters*, 414, 521-526 (1997); Lauwereys et al., *EMBO J.*, 17, 3512-3520 (1998); Decanniere et al., *Structure*, 7, 361-370 (1999)). By creating polyclonal libraries, many highly soluble sdAbs with high affinity and specificity have been isolated. However, it has been questioned whether sdAbs with desired affinity and defined conformations can be generated in the absence of prior immunization, i.e., with a naïve library (Lauwereys et al., *supra*). Immunization of domesticated valuable animals, such as camelids, raises serious ethical implications related to experiments with animals. Moreover, this approach has serious drawbacks because most of the pathogenic antigens cannot be injected into camelids, as this could endanger their lives. Considering the above drawbacks and limitations of the prior art, there exists a strong need for the generation of phage display libraries of sdAb antibody fragments derived from naïve libraries of camelid antibodies, in particular sdAb fragments of camelid heavy chain antibodies, which libraries may become a universal source of sdAbs for *in vitro* selection against any antigen of interest as a target. By choosing antigen targets located in tissues of therapeutic or diagnostic interest or importance, such libraries may provide new vectors for targeted delivery of therapeutic and diagnostic agents. Of particular interest to the present invention are antibody fragments targeting antigens of the endothelial tissue of the blood-brain barrier (BBB), which fragments may be used for the delivery of therapeutic and diagnostic agents into neuronal tissues.

25

The effective delivery of molecules into neuronal tissues remains one of the most perplexing challenges facing the pharmaceutical and biotechnology industries. The brain is isolated from the rest of the body by a specialized endothelial tissue known as the blood-brain barrier (BBB). The endothelial cells of the BBB are connected by tight junctions and efficiently prevent many therapeutic compounds from entering the brain. In addition to low rates of vesicular transport, one specific feature of the BBB is the existence of

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- enzymatic barrier(s) and high level(s) of expression of ATP-dependent transporters, including P-glycoprotein (Gottesman et al., *Ann. Rev. Biochem.*, **62**, 385-427 (1993); Watanabe, T., *Acta Oncol.*, **34**, 235-241 (1995)), which actively degrade/extrude various pharmaceuticals from the brain (Samuels B.L., *J. Clin. Pharmacol. Ther.*, **54**, 421-429 (1993). As a result, a plethora of compounds with demonstrated efficacy *in vitro* cannot be used as brain-targeting pharmaceutical agents *in vivo* unless appropriate delivery vehicles capable of overcoming the impermeability of the BBB are employed.
- 10 Only small (<600 Daltons) and hydrophobic (Pardridge, W.M., *Adv. Drug Delivery Reviews*, **15**, 5-36 (1995)) molecules can easily pass the BBB, a constraint that places enormous restrictions on drug development strategies. Current brain drug delivery practices either employ invasive neurosurgical procedures or non-invasive strategies such as pharmacological methods to
- 15 facilitate transport of drugs *via* intercellular or transcellular routes. In addition to invasive and highly limited neurosurgical strategies (e.g., intraventricular drug infusion, cerebral implants) and osmotic BBB opening applied clinically, strategies based on 1) physiological- and 2) pharmacological modulation of BBB permeability are being developed..
- 20 Strategies based on physiological approaches to drug delivery through the BBB use pseudonutrients that are substrates for BBB nutrient carrier systems (Pardridge, W.M., *supra*). At least eight different nutrient transport systems have been identified in cerebromicrovascular endothelial cells (i.e., glucose
- 25 transporter, the neutral amino-acid carrier, the basic amino acid carrier, the monocarboxylic amino acid carrier, the purine nucleoside transport carrier, the purine base carrier, choline carrier, and glutamate transporter) many of which are being exploited to carry drugs that 'mimic' the respective natural ligands for these transporters into the brain. While this strategy constitutes a
- 30 clear advance over current alternatives, it is limited by the fact that such drugs will have to compete with endogenous substrates normally transported by these systems.

For the pharmacologically-based strategies, the delivery of small molecules through the BBB include also lipidization approaches and liposomes (Pardridge, W.M., supra). Lipidization of small molecules involves chemical
5 modification of hydrogen bond-forming polar functional groups with apolar functional groups, e.g., O-methylation, or O-acetylation. The alternative approach is to attach free-fatty acyl or cholesterol groups to drugs in order to form more hydrophobic and BBB permeable compounds. The entrapment of various compounds into liposomes has been widely utilized to deliver drugs to
10 various tissues and organs. However, despite many efforts invested into developing liposomal strategies to overcome the BBB, liposomes have, in general, failed to improve the penetration of drug(s) into the brain (Micklus et al., *Biochim. Biophys. Acta*, **1124**, 7-12 (1992); Gennuso et al., *Cancer Invest.*, **11**, 118-128 (1993)). In fact, it has been shown that even small
15 liposome vesicles (50 nm) do not undergo significant BBB transport (Micklus et al., supra). Moreover, one novel pharmacological strategy to transiently disrupt the BBB takes advantage of the fact that the activation of specific peptide and/or neurotransmitter receptors expressed on cerebrovascular endothelial cells (CEC) leads to transient 'loosening' of
20 the tight junctions maintaining barrier integrity (Black, K.L., *Adv. Drug Delivery Reviews*, **15**, 37-52 (1995)). This strategy, known as receptor-mediated permeabilization, has been successfully used by Alkermes Inc. to deliver anti-tumor drugs into brain tumors by selectively disrupting the blood-tumor barrier with bradykinin B₂ receptor agonists (Inamura et al., *J. Cerebral Blood Flow*
25 *Metab.*, **14**, 862-870 (1994)). However, it appears that B₂ receptor activation does not affect BBB properties outside the peritumoral areas, and therefore this strategy appears to be ineffective in delivering drugs across the intact BBB (Inamura et al., supra).

30 The development of efficient ways to deliver large molecules such as peptides, proteins and nucleic acids across the BBB is also crucial to the future success of growth factor- and gene-based therapies to fight disorders

- of the central nervous system (CNS). The principal strategy currently being pursued to deliver these macromolecules across the BBB is the development of chimeric peptides (Pardridge, W.M., *Adv. Drug Delivery Reviews*, **15**, 109-146 (1995); Boado, R.J., *Adv. Drug Delivery Reviews*, **15**, 73-107 (1995)).
- 5 This strategy takes advantage of various receptors present on brain capillary endothelium that mediate the transcytosis of essential proteins through the BBB, including transferrin, insulin growth factor and low-density lipoprotein (Friden, P.M., in: *The Blood Brain Barrier: Cellular and Molecular Biology* (Pardridge, W.M., Ed.), Raven Press, New York, pp. 229-248 (1993)). This
- 10 process is known as a receptor-mediated endocytosis/transcytosis. Therefore, macromolecule delivery to the brain can potentially be achieved by coupling proteins and nucleic acids to agonist/antibody "vectors" which bind these receptors, allowing absorptive or receptor-mediated transcytosis to bring these compounds to the brain. Proof of principle for this technology has
- 15 been recently achieved using an anti-transferrin receptor antibody (OX-26) to successfully deliver endorphin, vasoactive intestinal peptide and BDNF into brain tissue in experimental animals (Pardridge, W.M., *supra*). Similarly, the same antibody has been used to deliver oligonucleotides and plasmid DNA (Boado, R.J., *supra*) into the brain parenchyma. However, before becoming a
- 20 useful therapeutic tool, chimeric peptide technology requires further development in the following areas: 1) the discovery of additional suitable vectors expressed on human BBB endothelium; and 2) the development of improved strategies to link vectors to proteins/nucleic acids.
- 25 The relative inability of polypeptides and polynucleotides to access the brain is compounded by the fact that even if they were to penetrate the BBB, the transport of these compounds across neuronal cell membranes is extremely low. Even direct intracerebral administration of peptides, antisense oligonucleotides and plasmid DNA often fail to produce the desired
- 30 therapeutic effect due to minimal diffusion and low uptake of these compounds into neurons and other cells of the CNS. To date, viral vectors have exhibited the highest levels of gene transfer efficiencies. However, the potential advantages offered by non-viral transfection systems, such as the

lack of viral gene elements, higher safety and lower immunogenicity, have fueled the development of non-viral alternatives for *in vivo* gene therapy (Hanania et al., *Amer. J. Med.*, **99**, 537-552 (1995); Gregoriadis, G., *TIBTECH*, **13**, 527-537 (1995)). For example, highly efficient *in vitro* gene transfer capacity has been reported for cationic liposomes (Gao et al., *Gene Therapy*, **2**, 710-722 (1995)), although their eventual utility may be limited by the fact that these "vectors" are quite toxic and are strongly inhibited by serum. More recently, polycationic non-lipid compounds have been shown to achieve superior gene transfer efficiencies *in vivo* relative to cationic liposome preparations (Goldman et al., *Nature Biotechnology*, **15**, 462-466 (1997)). However, attempts to deliver polypeptides and polynucleotides into neurons by complexing them with liposomes, nanoparticles, and low molecular weight surfactants have been largely disappointing because of the high intrinsic sensitivity of neurons to the toxic effects of such delivery systems (Abbott et al., *Mol. Med. Today*, **3**, 106-113 (1996)). In reality, even if these delivery systems were to effectively deliver drugs across neuronal and glial membranes, the "Achilles heel" remain their incapacity to penetrate the intact BBB. That formidable and persistent problem remains the predominant issue.

20

It is clear in view of the above that new approaches are necessary to identify and provide vectors capable of transigrating the BBB barrier and delivering therapeutic or diagnostic molecules to neuronal tissues. The present invention provides such new vectors free of many prior art limitations.

25

SUMMARY OF THE INVENTION

The present invention has overcome some of the above-discussed prior art limitations by generating a large size (in the order of 10^9) phage display library of antibody fragments of a non-immunized llama, which fragments comprise at least a part of the variable heavy domain (VH or V_HH domain) of llama antibodies. In a preferred embodiment, the fragments consist essentially of

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the variable heavy domain (VH or V_HH) of llama antibodies (sdAb fragments). The library has a number of unique features which distinguish it from similar libraries generated from other camelids.

- 5 From this library, sdAb fragments capable of binding selectively to human cerebromicrovascular endothelial cells (HCEC) and of transmigrating across an *in vitro* BBB model consisting of HCEC monolayer grown on porous artificial support dividing two media compartments have been isolated and proven to be targeting the brain *in vivo*. Due to their small size, selectivity of
- 10 binding to brain endothelium and ability to cross the BBB, these antibody fragments are useful not only as vectors for the delivery of therapeutic and diagnostic agents into the brain, but also as molecular templates for designing drug and gene delivery vectors targeting the central nervous system.
- 15 Thus, according to one aspect, the present invention provides a process for enriching a phage display library of antigen-binding antibody fragments derived from llama antibodies with phage species binding selectively to human endothelial cells expressing BBB antigens, each antigen-binding fragment comprising at least a part of the variable heavy domain (V_HH or VH)
- 20 of a llama antibody, said process comprising the steps of: (a) adsorbing the phage display library on first endothelial cells which do not express BBB antigens, (b) adsorbing phage species unbound to the first endothelial cells on second endothelial cells which express BBB antigens, and (c) stripping and recovering phage species bound to the second endothelial cells.

25

- According to another aspect, the invention provides a process for the preparation of a human blood-brain barrier (BBB) transmigrating antibody fragment derived from a llama antibody, said process comprising the steps of:
- 30 (a) providing a phage display library of antigen-binding antibody fragments derived from llama antibodies, (b) enriching the library for phage species selectively binding to endothelial cells expressing BBB antigens, (c) recovering from the enriched library phage particles selective to the endothelial cells expressing BBB antigens by comparing their binding to

various endothelial cells from peripheral and brain tissues, (d) enriching the library for phage species internalized into the endothelial cells expressing BBB antigens, (e) identifying in the enriched libraries from steps (b) and (d) phage particles transmigrating across an *in vitro* BBB and recovering such
5 phage particles, (f) comparing nucleotide sequences of DNA of phage particles recovered in steps (c) and (e) and selecting particles having both high selectivity to the endothelial cells expressing BBB antigens and transmigrating across the *in vitro* BBB, (g) amplifying DNA of the phage particles identified in step (f) and cloning into an expression vector a piece of
10 DNA coding for the antibody fragments displayed by the phage particles, (h) transforming host cells with the expression vector, (i) incubating the transformed cells under conditions allowing the expression of the cloned piece of DNA, and (j) recovering the antigen-binding antibody fragment so prepared.

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According to still another aspect, the invention provides an antibody fragment derived from a llama antibody, said fragment capable of transmigrating across the human blood-brain barrier (BBB) and selectively binding to an antigen on the surface of mammalian cells expressing BBB antigens.

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According to yet another aspect, the invention provides a therapeutic or diagnostic agent, said agent comprising an antibody fragment derived from a llama antibody, said fragment capable of selectively binding to an antigen on the surface of mammalian cells expressing BBB antigens and transmigrating
25 across the human blood-brain barrier (BBB), said antibody fragment being linked, directly or indirectly, covalently or non-covalently, to a therapeutic or diagnostic entity.

Other advantages, objects and features of the present invention will be readily
30 apparent to those skilled in the art from the following detailed description of preferred embodiments in conjunction with the accompanying drawings and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of a typical four-chain IgG-type immunoglobulin (antibody) showing (a) the structure and arrangement of heavy and light chains and the approximate positioning of interchain disulfide bonds, and (b) the organization of the antibody molecule into paired domains.

Fig. 2 is a schematic representation of various modifications and fragments of IgG-type antibodies, and antigen-binding fusion proteins derived from such fragments.

Fig. 3 is a schematic representation of steps involved in construction of the phage display library of llama sdAb antibody fragments according to the present invention. For simplicity, only the coding sequences of the mRNA transcripts are shown. A, a: heavy chain mRNA of conventional four-chain (A) and two-chain heavy chain (a) antibodies; B, b: RT-PCR product derived from A and a, respectively; c: V_HH derived from heavy chain antibodies. Variable heavy (V_H and V_{HH}) and constant heavy ($CH1$) domains are marked with dark and light shading, respectively.

Fig. 4 is a schematic representation of steps involved in selection of blood-brain barrier-binding sdAbs from llama sdAb phage-displayed library. A) Subtractive panning protocol of llama single-domain antibody phage-displayed library against human lung microvascular cells (HLMEC) and human cerebromicrovascular endothelial cells (HCEC) used. Clones that bound selectively to HCEC were recovered by three stripping washes, and those internalized by HCEC were recovered by cell lysis. Phage recovered from S3 and internalized fractions were amplified and used as input library for each subsequent round of panning. Total of four panning rounds was performed. B) Functional selection of phage clones from the enriched library was done based on two criteria: a) selectivity and high affinity binding to HCEC (left branch), determined by phage binding to HCEC and peripheral endothelial cells by phage ELISA, and b) the ability of phage clones to

transmigrate across *in vitro* BBB model (right branch). Only phage clones with full V_HH inserts fulfilling both functional criteria were chosen for further characterization.

- 5 Fig. 5 is a graph showing binding of selected phage-displayed sdAb clones to endothelial cells. Relative binding affinity of 16 phage clones with full-length V_HH inserts to HCEC (black squares), HLMEC (black triangles), and HUVEC (open circles) was determined by ELISA against phage coat protein P8. Cells grown in 96-well plates were exposed to the same amount of each phage clone for 1 h, and after washing the bound phage was detected by ELISA. The absorbance values were normalized for non-specific binding (n.s.).

- Fig. 6 is a schematic and a graph showing the *in vitro* BBB model used and transmigration of enriched llama sdAb phage-displayed library across the *in vitro* BBB model. A) *In vitro* BBB model consists of human cerebromicrovascular endothelial cells (HCEC) grown as a monolayer on a semipermeable membrane of the tissue culture insert. HCEC are exposed to media conditioned by fetal human astrocytes (FHAS-CM) applied to the bottom compartment. To estimate transcellular passage, phage was added to the upper chamber and aliquots from the bottom chamber were collected over various periods of time. B) 10¹¹ transducing phage units of the wild type phage (open bars), phage displaying an unrelated sdAb (NC11) (grey bars), and phage library enriched for high affinity HCEC binding and HCEC internalizing phage (black bars) were applied to the top chamber of triplicate BBB assemblies and the phage titre was determined in aliquots of the bottom chamber at the indicated time points.

- Fig. 7 is a graph showing the binding of sdAb fusion proteins to endothelial cells. Binding of FC44 (black bars), FC5 (grey bars), and NC11 (open bars) - c-myc-His₅ fusion proteins to (A) cell lysates of human cerebromicrovascular endothelial cells (HCEC), human pial artery endothelial cells (HPEC), human

umbilical vein endothelial cells (HUVEC), human lung microvascular endothelial cells (HLMEC), and fetal human astrocytes (FHAs), and (B) to live HCEC and HLMEC. The binding was determined by ELISA against c-myc. Each bar represents the mean \pm S.E.M. of 6 wells in one representative experiment out of three independent experiments.

Fig. 8 is a photograph illustrating the binding of sdAb fusion proteins to brain endothelial cell fractions and uptake into brain endothelial cell. The antibodies were labeled with Alexafluor 488/532 and applied to vesicular or membrane fractions or to live cells for 2 h. After washing to remove unbound antibodies, fluorescence bound to cellular fractions or taken up by cells was detected using confocal or fluorescence microscopy. Panels show binding of the fluorescently labeled FC44, FC5, and NC11 c-myc-His₅ fusion proteins to (A) endocytic vesicles and (B) membranes isolated from human cerebrovascular endothelial cells (HCEC), and (C) the uptake of these antibodies into live HCEC.

Fig. 9 is a graph showing the transmigration of sdAb fusion proteins across the *in vitro* blood-brain barrier model. Panels show clearances of (A) 10 kDa radiolabeled dextran and (B) FC5, FC44, and NC11 c-myc-His₅ fusion sdAbs across the *in vitro* human BBB model. A) 10 kDa dextran was added to the top chamber and fluorescence intensity was determined in aliquots collected from the bottom chambers of triplicate empty membranes (open circles) and membranes covered by HCEC monolayers (closed circles) at the indicated time points. B) 100 μ g of FC5 (gray circles), FC44 (black circles), or NC11 (open circles) was added to the top chamber of the BBB model, and concentrations of the antibodies in aliquots collected from bottom chambers at the indicated time points were determined using a nickel-trap ELISA. Clearance values were determined as described. Each point represents the mean \pm S.E.M. of clearance values of 3 membranes in one representative experiment out of three independent experiments.

Fig. 10 is a graph and a photograph showing the biodistribution of llama sdAb libraries and selected sdAb fusion proteins in mice. A) *In vivo* distribution of phage in various organs of mice injected with 10^9 transducing phage units of the wild type phage and phage carrying FC5, or FC44 sdAb. The mice were perfused transcardially 4 hours after the phage injection, and phage titers in the brain, lung, kidney and liver were determined. Each bar is the mean \pm S.D. of phage titres determined from 3 animals. B) Biodistribution of the FC5, FC44, and NC11-c-myc-His₆ fusion proteins in various organs of mice after i.v. injection of 30 μ g/kg of respective sdAbs. The organs were harvested 4 h after injection and sdAbs were extracted by affinity purification on a Ni²⁺ column and detected by immunoblot against c-myc tag. The blot is representative of the results obtained from 3 animals.

Fig. 11 is a photograph showing the affinity purification of the antigen/sdAb complex from HCEC. A) A gradient elution of the antigen/antibody complex with increasing strengths of imidazole buffer. Denatured FC44/antigen complex was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane, and antigen was detected by probing the membrane with FC44 fusion protein and subsequently with anti-c-myc antibody as described in the Experimental Procedures. B) Silver stained gel of respective resolved FC44, FC5, and NC11 antigens eluted from the Ni²⁺ affinity column with 400 mM imidazole, C) Identical gel as in B) transferred to nitrocellulose membrane and probed with phage-displayed FC44 followed by anti-phage coat protein P8 antibody. Gels shown are representatives of at least three experiments showing similar results.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the following, positions of amino acid residues in antibodies and antibody fragments are indicated according to the Kabat numbering.

The present invention uses a large size (in the order of 10^9) phage display library of single-domain fragments of variable heavy domains (V_H and V_{HH}) of llama antibodies. The library, which has been generated using lymphocytes of a non-immunized animal (naïve library), can be used for *in vitro* selection
5 against any antigen of interest as a target. The size of the library makes it highly probable that an antibody specific to the intended target will be identified among the library's sdAb fragments.

The choice of a naïve library as the source of llama antibodies was based in
10 part on the fact that the immune system of camelids has evolved over time in harsh environments and that its unique physiological and morphological features have helped the camelids to withstand water scarcity, adapt to climate extremes and develop a natural resistance to deadly viral diseases. The sero-epidemiological studies have confirmed that camelids produce
15 antibodies to a great number of pathogenic viruses without developing the disease (Werney et al., Infectious Diseases of Camelids, Blackwell's Wissenschaft Verlag, Berlin (1995)). This means that antibodies of therapeutic importance can be isolated from the antibody repertoire of camelids without prior immunization with potentially dangerous pathogens or
20 fragments thereof.

Among the camelids, llama is the smallest animal which can survive in a severe, cold climate. Lymphocytes of a llama from a farm located in Osgoode (Canada) have been used to generate the phage display library of sdAbs of
25 heavy chain antibodies. From this library, dAbs binding selectively binding to human cerebrovascular endothelial cells (HCEC) have been isolated. These sdAbs prove to target the brain *in vivo* and transmigrate the BBB.

CONSTRUCTION OF A NAÏVE LLAMA sdAb PHAGE DISPLAY LIBRARY

30 Fig. 3 depicts a schematic representation of steps involved in the construction of the V_{HH} -derived sdAb phage display library. As the first step, lymphocytes from the fresh blood of llama (from a farm located at Osgoode, Ontario,

Canada) were prepared and their RNA was isolated using techniques well known to those skilled in the art. RT-PCRs (reverse transcriptase-polymerase chain reactions) were performed using primers annealing at the 5' end of V_H or V_HH and CH2 genes of IgG. The amplified products were separated and
5 fragments of the expected size derived from conventional IgG (~900bp) and heavy chain IgG (~600bp) were observed on the agarose gel. The smaller fragment was gel purified and used in a second PCR to amplify the V_HH genes. The amplification products were cloned into fd-tet (GIID) vector, between the leader signal and gene III, to produce fusion proteins, which
10 were displayed on the filamentous phage particles using a modified procedure.

As is well known to those skilled in the art, the probability of isolating a protein with high affinity or specificity against a target (antibody) of interest increases
15 with the size of the library. Generally, two different types of vectors are used for generating phage display libraries: phagemid vectors and phage vectors. Libraries having size in the order of 10^8 can be constructed with relative ease using phagemid vectors. However, a phagemid-based libraries suffers from some serious drawbacks. First, phagemid vectors provide typically a
20 monovalent display and therefore may not select for lower binding (of lower affinity), but potentially important antibody fragments. Second, a phagemid-based library allows for the enrichment of phage particles displaying deleted versions of the antibody fragments. Such particles, often with no binding activity, are preferably selected during the panning process over those
25 displaying the full-length fragments and therefore obscure the process of selection of the full-length binders. Third, constructing a phagemid-based library requires a helper phage and therefore library construction, panning and downstream phage binding assays become a far more complicated and tedious task. For these reasons the use a phage vector for the library
30 construction is preferred.

20

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One of the most widely used phage vectors is fd-tet (Zacher III et al., *Gene*, 9, 127-140 (1980)) which consists of fd-phage genome, plus a segment of Tn10 inserted near the phage genome origin of replication. Tn10 contains a tetracycline resistance gene, tetA, and thus confers tetracycline resistance to the host cells carrying the fd-tet vector. It has often been observed that the size of the fd-tet based library was generally low (in the range of $10^5 - 10^6$) (Harrison et al., *Methods in Enzymology* [Ed. Abelson, J.N.], 267, 83-109 (1996); Krebber et al., *FEBS Letters*, 377, 277-331 (1995)), possibly due to the toxic effect of tetA gene product on the host cells. According to the modified procedure of the present invention, the library was propagated as plaques in the absence of tetracycline, resulting in a llama V_HH library of size of approximately 8.8×10^8 . This is the largest size library ever obtained using fd-tet vector. Due to its size, the library has an enhanced probability of selecting therefrom proteins (antibody fragments) binding to almost any given target (antigen).

It would be known to those skilled in the art that, at least in principle, the display library of the invention could be generated using vectors other than phages, such as bacteria (e.g., *E. coli*) (Daugherty et al., *Protein Eng.*, 613-621 (1999); Georgiou et al., *Nat. Biotechnol.*, 29-34 (1997)) or yeast (e.g., *Saccharomyces cerevisiae*) (Kieckhefer et al., *Proc. Natl. Acad. Sci. USA.*, 5651-5656 (1999); Kieckhefer et al., *Protein Eng.*, 1303-1310 (1997); Cho et al., *J. Immunol. Methods*, 179-188 (1998); Boder et al., *Nat. Biotechnol.*, 553-557 (1997)). Obtaining large libraries, comparable in size to phage display libraries, is, at least in theory, possible using these vectors. However, these display systems have not been of a widespread use, as they require expensive flow cytometry cell sorting instruments for selection. In addition, the *E. coli* display system is not suitable for panning against large macromolecules, such as proteins, due to the interference of the lipopolysaccharide layer of *E. coli* with the binding process (Boder et al., supra). Surface display of an scFv on mammalian cells has also been reported (Rode et al., *J. Immunol. Methods*, 151-160 (1999); Rode et al.,

BioTechniques, 650, 652-656, 658 (1996)). However, no antibody library has been so far constructed using vectors other than phages, as the construction and screening in these alternative display systems are not as rapid or versatile as for phage display libraries.

5

SEQUENCE ANALYSIS

Colony PCR of 80 randomly selected clones showed that more than 60% had the full-length V_HH genes (sdAbs). The identity of the VL interface amino acids at position 44, 45 and 47 as well as the CDRs sequence of 28 randomly
10 selected dAbs have been determined and are summarized in Table 1. Similarly to published results, the majority of the CDRs of the sequenced dAbs are 13-17 amino acid long, demonstrating that the llama sdAb library of the invention is derived from heavy chain antibodies. However, the present library is distinct in several aspects from the known V_HH libraries.

15

Previously generated camelid sdAb libraries were characterized by typical presence of Glu, Arg and Gly in positions 44, 45 and 47, respectively, of the VL interface of V_HH domain. The occurrence of cysteine at position 45 was also frequent in V_HH, as opposed to VH domain of four-chain IgGs. The
20 present library, as shown by sequence analysis, lacks these characteristics, as only one sdAb (C35) has Glu44, Arg45 and Gly47. Five sdAbs (C1, C29, C43, C44, and C48) are characterized by Gly44, Leu45 and Trp47, the very same residues which are highly conserved in the VL interface of VH domain of conventional four-chain antibodies. The majority of sdAbs of the present
25 library have Arg in position 45 of the VL interface. This occurrence of Arg45 is not unique to camelid V_HH, as a number of conventional antibodies, such as H1-I6 (VH) and V13 (VH), have been found to have Arg in position 45 (Blier et al., *J. Immunol.*, **139**, 3996-4006 (1987); Crews et al., *Cell*, **29**, 59-66 (1981)). The presence of Gly at position 35 was observed to always accompany Phe
30 at position 37, unlike a previously reported llama library in which this pairing was observed in only 50% of the sequences. This is noteworthy in view of the fact that Gly at position 35 results in local conformational changes that allow

Trp101 to stack with Arg45 in addition to engaging in aromatic-aromatic interactions involving Phe37 and Trp103. For the present library, 12 of 27 sdAbs have Trp at position 52a, whereas only 1 of the 51 previously published sequences have Trp at this position.

5

Another major difference between the present library and the previously reported V_HH libraries of camelids concerns the CDR cysteines. Previously generated libraries were characterized by a high incidence of cysteine pairs in CDRs, whereas none of the 28 sdAbs (Table 1) of the present library had any
10 cysteine in their CDRs. The library of the invention is therefore characterized by a very low presence or by the absence of cysteine residues in CDRs.

Finally, the present library, which was designed and constructed to contain only antibody fragments consisting of variable heavy chain domains (V_HHs),
15 also contains a substantial number of typical conventional variable heavy domains (VHs) (for example, sdAbs C1, C29, C43, C44 and C48 of Table 1, some sdAbs of Table 2). This contamination is most likely the results of PCR cross-overs between the VHs and V_HHs during the step of RT-PCR (Figure 3) (Tomlinson et al., *J. Mol. Biol.*, **227**, 776-798 (1992); Muyldermans et al.,
20 *Protein Eng.*, **7**, 1129-1135(1994)). These VHs are genuine antigen binding fragments, as shown in Table 2, produced in high yield in *Escherichia coli*. They are highly soluble, have excellent temperature stability profiles and do not display any aggregation tendencies (Tanha et al., manuscript in preparation; Vranken et al., submitted). The very close similarity of these
25 molecules to human VHs makes them potentially very useful as therapeutic sdAbs.

For the library of the invention, amino acids of the VL interface are most frequently:

- 30 at position 44 – Gly, Glu, Gln, Lys, Ala and Asp,
 at position 45 – Leu, Phe, Pro and Arg, and
 at position 47 – Trp, Tyr, Phe, Leu, Ile, Val and Gly.

SELECTION OF ANTIBODY FRAGMENTS BINDING SELECTIVELY TO HCEC

5 **1. Enrichment of phage displayed llama single-domain antibody (sdAb) library for HCEC specific antibodies by subtractive panning**

Phage display libraries of peptides, proteins and antibodies have previously been used to identify 'binders' to biological targets *in vitro* and *in vivo* (Hoogenboom et al., *Immunotechnology*, 4, 1-20 (1998)), including purified
10 antigens, whole cells and tissues. Previous work using *in vivo* panning of phage displayed peptide library resulted in identification of organ specific peptide sequences in brain and liver (Pasqualini et al., *Nature*, 380, 364-366 (1996)). However, the brain targeting peptides were 'trapped' on the surface of blood capillaries and did not cross the BBB (Pasqualini et al., *supra*).
15 Unlike peptides, antibodies are far more stable inside the cell and some antibodies have been shown to transmigrate the BBB *via* receptor-mediated transcytosis (Pardridge W.M., *J. Cerebral Blood Flow. Metabol.*, 17, 713-731 (1997)). The antibodies according to the present invention are the smallest possible antigen binding antibody fragments that selectively bind to and
20 transmigrate across human cerebrovascular endothelial cell (HCEC) monolayers and have properties of a brain delivery vector.

To select suitable fragments, inventors used the above-disclosed naïve llama sdAb phage displayed library derived from the V_HH of the heavy chain IgGs
25 which occur naturally in the absence of light chain (Muyldermans et al., *J. Mol. Recog.*, 12, 131-140 (1999)). The average molecular weight of these sdAbs is 13kDa, approximately half the size of a scFv. As discussed above, the library (about 8.8×10^8 species) exists in a filamentous phage vector and is propagated as plaques in the absence of antibiotics. Characterization of
30 more than a dozen sdAbs isolated from this library has shown K_ds in the low micromolar range (data not shown). Two features of this library are of particular importance for isolating antibodies with the ability to penetrate

and/or transmigrate cell layers. First, the library is constructed using a phage vector, as opposed to a phagemid vector, and therefore the display of sdAbs is multivalent. The sdAbs in the multivalent presentation mode favor receptor cross-linking and phage internalization (Becerril et al., *Biochem. Biophys. Res. Commun.*, **255**, 386-393 (1999)) and are more effective in capturing and retaining 'binders' during the course of panning. The affinity of such sdAbs can easily be improved by mutagenesis because they are derived from a naïve repertoire and have not gone through *in vivo* affinity maturation. The second important feature of this library is the small size of sdAbs (~13kDa as compared to 150kDa for an IgG), a preferred choice for macromolecules required to penetrate dense tissues such as the BBB (Muyldermans et al., *supra*).

The subtractive panning used to enrich for BBB-specific sdAbs from the naïve phage displayed library is schematically shown in Fig. 4, part A. Two endothelial cell types were used for subtractive selection: human lung microvascular endothelial cells (HLMEC) and human cerebromicrovascular endothelial cells (HCEC). It was previously shown that HCEC express BBB-specific antigens (e.g., γ -glutamyl transpeptidase, P-glycoprotein, HT7, occludin, etc.) that are not expressed in peripheral endothelial cells, including HLMEC (Stanimirovic et al., *J. Cell Physiol.*, **169**, 455-467 (1996)).

In order to identify BBB-selective sdAbs, the naïve phage library was preabsorbed onto HLMEC to remove non-specific, common endothelial binders, and then applied to HCEC. After removing unbound phage, phage bound to HCEC was dislodged by three rounds of highly stringent stripping washes (S1, S2 and S3) to favor the selection of sdAbs with higher affinity. HCEC were then lysed to capture the internalized phage (Int). PCR on 80 individual plaques from S1, S2, S3 and Int fraction revealed that the output phages from S3 contained the highest percent of clones with the full-length V_HH insert. The phage in S3 fraction was then amplified and used as an input phage for second round of panning. Four rounds of panning were

performed and for each round the input phage was derived from the fraction which showed the highest percent of phage with full-length V_HH insert.

The progress of panning was monitored by phage titer measurements, PCR and phage ELISA. As shown in Table 2, the phage titers bound to the HLMEC cells decreased with each subsequent round of panning while those bound to HCEC dropped after the first round and then progressively increased from the second to fourth round of panning, indicating enrichment for HCEC-specific sdAbs. After fourth round of panning, fractions S1, S2, S3 and Int contained 80%, 80%, 70% and 30%, respectively, of clones with the full-length V_HH insert. The remaining clones either had no inserts or possessed one or two of the CDR domains and framework regions.

Table 2. Phage titers determined after various steps of subtractive panning procedure.

Panning Rounds		1	2	3	4
20	Phage bound to HLMEC	6.0x10 ¹¹	1.9x10 ¹¹	0.6x10 ¹¹	0.08x10 ¹¹
	Phage applied onto HCEC	4.0x10 ¹¹	8.1x10 ¹¹	9.4x10 ¹¹	9.92x10 ¹¹
25	Phage bound to HCEC				
	Strip 1	2.1x10 ⁸	1.3x10 ⁷	1.3x10 ⁷	
	Strip 2	0.2x10 ⁷	0.9x10 ⁷	1.1x10 ⁷	
	Strip 3	0.9x10 ⁶	0.9x10 ⁶	4.4x10 ⁷	
30	HCEC cell lysate (internalized)	0.7x10 ⁷	0.1x10 ⁷	4.2x10 ⁷	

Table 3. Sequences of phage-displayed sdAbs clones that transmigrated across the human *in vitro* blood brain barrier model.

	1	10	20	30	
FC5	E V Q L Q A S G G L V Q A G G S L R L S C A A S G F K I T H Y T M				
FC44	E V Q L Q A S G G L V Q A G G G L R L S C S A S V R T F S I Y A M				
FC7	E V Q L Q A S G G L V Q A G G G L R L S C S A S V R T F S I Y A I				
FC5	G W F R Q A P G K E R E F V S R I T W G G D N T F Y S N S V K G R F	40	50	60	
FC44	G W F R Q A P G K E R E F V A G I N R S G D V T K Y A D F V K G R F		a		
FC7	G W F R Q A P G K E R E F V A G I N R S G D V T K Y A D F V K G R F				
FC5	T I S R D N A K N T V Y L Q M N S L K P E D T A D Y Y C A A G S T S	70	80	90	
FC44	S I S R D N A K N M V Y L Q M N S L K P E D T A L Y Y C A A T W A Y		a b c		
FC7	S I S R D N A K N M V Y L Q M N S L K P E D T A L Y Y C A A T W A Y				
FC5	T A T P L R V - - D Y W G K G T Q V T V S S	100 a b c d e f g h		113	SEQ ID NO: 85
FC44	D T V G A L T S G Y N F W G Q G T Q V T V S S				SEQ ID NO: 86
FC7	D T V G A L T S G Y N F W G Q G T Q V T V S S				SEQ ID NO: 87

10¹² phage was preabsorbed onto HLMEC and unbound phage was then applied to HCEC. Bound phage was dislodged by three stripping washes. Internalized phage was harvested by cell lysis. Second and third rounds of panning were started with 10¹² of amplified phage from strip 3 of the previous panning. The fourth round of panning was started with 10¹² of amplified phage from strip 1 of the third panning.

2. Selection of candidate sdAbs from the enriched llama sdAbs phage displayed library based on functional criteria

Further selection of sdAb species from the library enriched for HCEC specific binders obtained by subtractive panning was done using two functional criteria: i) selective binding to HCEC compared to peripheral endothelial cells, and ii) the ability of phage clones to transigrate across *in vitro* BBB model (Figure 1, part B).

2.1. Selection for specificity and higher affinity by phage ELISA

Following 4 rounds of subtractive panning, 58 clones with full-length V_HH inserts were identified by plaque PCR. Phage ELISA using all 58 clones revealed that 16 clones repeatedly showed a selective binding to HCEC as compared to HLMEC or human umbilical vein endothelial cells (HUVEC) (Fig. 5).

Sixteen clones that selectively bound HCEC identified by phage ELISA were subsequently sequenced and found to contain three different sequences (Table 3). Two sequences differed only at position 34 in CDR1 (Ile to Met) (Table 3) indicating that all 16 clones belong to only 2 sdAb species (Table 3), designated FC5 and FC44. These two sdAbs have 4 amino acids, Phe, Glu, Arg and Phe at positions 37, 44, 45 and 47 which are signature residues for llama V_HHs (Muyldermans et al., supra). Both sdAbs are characterized by a 17 residue CDR2 and the absence of additional Cys at positions 30-33 or 45,

hence, belonging to subfamily 1a (Nguyen et al., *EMBO J.* **19**, 921-930 (2000)). FC5 and FC44 showed no significant homology to each other in their CDRs suggesting that they recognize different antigenic epitopes.

5 **2.2. Selection of sdAbs with ability to transmigrate *in vitro* BBB model**

A separate round of selection was performed to select species capable of crossing *in vitro* BBB model (Fig. 4, part B). *In vitro* BBB model used for these studies consisted of a HCEC monolayer grown on a semi-permeable membrane positioned to separate two media compartments as shown in Fig. 10 6, part A. Media conditioned by fetal human astrocytes (FHAs) were used to induce the BBB phenotype of HCEC. The model has been characterized in detail previously (Muruganandam et al., *FASEB J.*, **13**, 1187-1197 (1997)). Similar *in vitro* BBB models using bovine (Dehouck et al., *J. Neurochem.*, **58**, 1790-1797 (1992); Pardridge et al., *J. Pharmacol. Exp. Therap.*, **253**, 884-891 15 (1990)) or porcine (Franke et al., *Brain Res.*, **816**, 65-71 (1999)) cerebral endothelial cells have been used to predict brain bio-availability of variety of compounds with different physico-chemical properties, including those transported across the BBB by energy-dependent mechanism(s).

20 Phage contained in the third stripping wash and internalized fractions of the fourth panning round was amplified and 10^{11} pfu was applied to the top chamber of the *in vitro* BBB model. Phage titers in the bottom chamber were determined after various time intervals. The integrity and tightness of HCEC monolayers were assessed by the diffusion of the small paracellular marker, 25 sodium fluorescein (Muruganandam et al., supra). The permeability coefficient (P_e) for sodium fluorescein was $[(3.2 \pm 0.3) \times 10^{-3}]$ cm/min, whereas HCEC monolayers were virtually impermeable for 70 kDa dextran (data not shown).

30 Both the wild type (i.e., empty) phage and the phage library displaying unrelated antibody (NC11) produced low phage titers in the bottom chamber 60-90 min after addition to the top compartment (Figure 6, part B). In contrast,

the amplified library enriched for HCEC binding and internalized phage clones produced high titers in the bottom chamber as early as 15 min after addition, and titers reached 5000-7000 pfu at 60-90 min (Figure 6, part B). Empty membrane did not restrict the passage of phage particles, as the titers in the bottom chamber were 15-40 x10⁴ pfu after 60-90 min of diffusion (data not shown). Plaque PCR of phage clones that had transmigrated across the *in vitro* BBB model between 15-30 min showed full-length (600 bp) V_HH inserts in 10% of all clones. Sequencing revealed that all phage clones with the full-length inserts contained FC5 or FC44 gene sequences. Plaque PCR and sequencing of full-length clones applied to the top chamber showed that this fraction, in addition to FC5 and FC44, contained clones with various other sequences.

Given the molecular weight ($\sim 1.4 \times 10^4$ kDa), the dimensions of filamentous phage particles (9X900 nm), and the complete barrier that HCEC monolayers presented for high molecular weight dextran, it was concluded that phage crossed the HCEC monolayer using a transcellular route. The transcellular phage transport was greatly facilitated by enriching phage library for species that confer tropism for HCEC binding and internalization. The mechanism by which phage enters mammalian cells and crosses cellular barrier(s) are poorly understood. Filamentous bacteriophage expressing the antibody against the growth factor receptor ErbB₂ has been shown to enter mammalian cells and express the reporter gene inserted in the phage genome (Poul et al., *J. Mol. Biol.*, **288**, 203-211 (1999)). It has been suggested that filamentous bacteriophage enters mammalian cells *via* receptor-mediated endocytosis (Becerril, et al., *supra*, Poul, et al., *supra*) and research addressing viral infections of the brain has demonstrated the ability of viral particles to transmigrate across the intact BBB (Banks et al., *J. Cell. Sci.*, **11**, 533-40 (1998)).

30

3. Functional characterization of FC5- and FC44-c-myc-His₅ fusion proteins

To assess functional properties, fusion proteins of FC5, FC44, and an unrelated anti-idiotypic antibody derived from the llama library (NC11; used as a negative control) with c-myc and His₅ tags were expressed in a bacterial expression system and purified. His₅ tag was used for the affinity purification on a Ni²⁺ column, and for immobilization of fusion proteins on a Ni²⁺-coated ELISA plate. C-myc tag was used to detect the sdAbs by ELISA and/or immunocytochemistry using HRP-conjugated anti-c-myc antibodies. Molecular weight of these fusion proteins was ~16kD.

10 3.1. Binding and uptake of fusion proteins in endothelial cells

Binding of the purified sdAbs to cell lysates of various endothelial cells derived from brain or peripheral organs, including HCEC, human pial artery endothelial cells (HPEC), HLMEC, HUVEC, and fetal human astrocytes (FHAs) demonstrated a selective binding of FC5 and FC44 to endothelial cells derived from the brain vasculature (i.e., HCEC and HPEC) (Fig. 7, part A). FC44 also bound to FHAs lysates (Fig. 7, part A), suggesting that antigen 'receptors' for this antibody may be expressed in brain cells other than endothelium. Binding of NC11 was at the background level. FC5 and FC44 also demonstrated a selective binding to live HCEC compared to HLMEC (Fig. 7, part B), while NC11 failed to bind either cell type.

FC5 and FC44 labeled with the fluorescent dye, Alexafluor 488, bound to both the vesicular (Fig. 8, part A) and membrane (Fig. 8, part B) fractions of HCEC. Furthermore, fluorescently labeled FC5 and FC44, but not NC11, were taken up by live HCEC (Figure 8, part C). No cellular toxicity of either sdAb was observed over 24 h period at 30 µg/ml (data not shown). Under the same experimental conditions, no uptake of FC5 and FC44 was detected in HLMEC (data not shown).

30 The uptake of FC5 and FC44 into HCEC was significantly reduced at 22°C, and completely abolished at 4°C (data not shown), while the uptake of lipid-

soluble diazepam was unaffected by temperature, suggesting that FC5 and FC44 are most likely internalized into HCEC by an energy-dependent pathway. The receptor-mediated endocytosis of transferrin/transferrin receptor complex has also been shown to be strongly suppressed by
5 hypothermia and/or energy deprivation (Tsuji et al., *Adv. Drug Delivery Rev.*, **36**, 277-298 (1999); Moos et al., *Cell. Mol. Neurobiol.*, **20**, 77-95 (2000)).

3.2. Transmigration of fusion proteins across *in vitro* BBB model

The ability of FC5 and FC44 fusion proteins to transmigrate across the HCEC
10 monolayers was investigated using *in vitro* BBB model described. The concentration of the sdAbs in the bottom chamber at given times was determined using a nickel-plate ELISA, and clearance values calculated as described (Muruganandam et al., *supra*). The HCEC monolayer was found to be virtually impermeable for 10 kD dextran (Fig. 9, part A) and for NC11
15 fusion protein (Fig. 9, part B). In contrast, both FC5 and FC44 fusion proteins crossed HCEC monolayer (Fig. 9, part B) showing clearance values comparable to those seen across empty membranes (2.28 ± 0.27 vs. 2.42 ± 0.15 $\mu\text{l}/\text{min}$ for FC5, respectively; 3.23 ± 0.62 vs. 2.51 ± 0.21 $\mu\text{l}/\text{min}$ for FC44, respectively; 0.097 ± 0.005 vs. 2.33 ± 0.27 $\mu\text{l}/\text{min}$ for NC11, respectively). The
20 clearance for both FC5 and FC44 was linear over 60 min (Fig. 9, part B) with the clearance of FC44 slightly higher than that of FC5 (Fig. 9, part B). FC5, FC44, or NC11 did not change *in vitro* BBB permeability as assessed by clearance of sodium fluorescein and measurements of the transendothelial electrical resistance. Thus, FC5 and FC44 fusion proteins retained the ability
25 to transmigrate across the *in vitro* BBB model, carrying across an intact c-myc-His₅ peptide tag.

4. In vivo brain homing of FC5 and FC44

30 To assess the ability of FC5 and FC44 to target brain in vivo, both phage-displayed FC5 and FC44 and soluble FC5 and FC44 fusion proteins were injected intravenously (i.v.) into mice, and the biodistribution in various organs

was analyzed after 4-6 hours using phage titre determination and Western blot detection, respectively.

Both phage-displayed FC44 and FC5 (Fig. 10, part A) produced significantly higher titers in the brain tissue than in the lung tissue, whereas wild type phage library produced no measurable titers in the brain and significant titers in the lung (Fig. 10, part A). Kidney and liver tissues produced high phage titers for FC44, FC5 and wild type phage libraries (Fig. 10, part A), suggesting that these organs are likely elimination/excretion routes for phage particles.

FC5, FC44, and NC11 fusion proteins were extracted from perfused mouse tissues 4-6 h after injection, using affinity purification on a Ni²⁺ column, and were detected on a Western blot using an anti-c-myc antibody (Fig. 10, part B). Both FC5 and FC44 fusion proteins, but not NC11 fusion protein, were detected in the brain tissue (Fig. 10, part B) after capillary depletion, as well as in the brain capillary fraction (data not shown). Kidney tissue showed strong bands for all three sdAb fusion proteins (Fig. 10, part B).

These results show that FC5 and FC44 sdAbs derived by *in vitro* subtractive panning and functional selection procedures have the ability to target the brain after i.v. injection *in vivo*, in a partly selective manner. These results also prove that small peptides can be attached to and transported along with FC5 and FC44 as a fusion protein(s) (MW of c-myc-His₅ tag ~3 kD; 26 amino acids) across the BBB *in vitro* and *in vivo*.

From the above, it would be obvious to those skilled in the art that antibody fragments of the present invention can be used as carriers (vectors) for therapeutic and diagnostic agents to be specifically delivered to the surface and/or the interior of the cerebral endothelial cells and/or across the blood-brain barrier. Such therapeutic or diagnostic agents include hydrophilic molecules, peptides, proteins, pieces of DNA, fluorescently or radioactively labelled compounds, phage particles, liposome formulations, polymer

formulations etc. Such agents can be attached to the antibody fragments either directly or indirectly (e.g., via suitable linkers), either by covalent or non-covalent bonds, for example by using complementary pieces of DNA attached to the antibody fragment and the molecule of a therapeutic or diagnostic agent.

5. HCEC antigen(s) recognized by FC5 and FC44

In order to characterize the nature of HCEC antigen(s) recognized by FC5 and FC44, the antigen/antibody complex was affinity purified using Ni^{2+} affinity column. Imidazole gradient washings eluted most of the FC44/antigen complex (Fig. 11, part A). The FC44 antigen was detected by separating FC44/antigen complex on a Western blot and by exposing the membrane to FC44-c-myc-His₅-fusion protein and subsequently to anti-c-myc antibody. The antigen recognized by FC44 appeared as a triple band of approximately 36 kDa (Fig. 11, part A). A band of the same molecular weight was also detected on the silver-nitrate stained SDS-PAGE gel (Fig. 11, part B), as well as on the immunoblot membrane probed first with the phage-displayed FC44 and subsequently with the anti M13-phage coat protein (P8) antibody (Fig. 11, part C). FC44 failed to recognize any epitope in either resolved FC5/antigen complex or NC11/antigen complex. FC5 antigen (Fig. 11, part B) could not be detected using this approach indicating a possible loss of structural epitope upon denaturation.

Although the exact identity of antigen(s) recognized by FC5 and FC44 is as yet unknown, the future identification and characterization of these antigens may prove important for understanding the molecular mechanisms of transcytosis across the BBB. Moreover, identification of antigen-recognition epitopes of FC5 and FC44 will allow for 'humanizing' and/or engineering these sdAbs to the minimal effective size and maximal affinity. FC5 and FC44 sequences can then be used as a template for molecular modelling of small drugs/ligands against their respective antigens.

FC5 and FC44 have several advantages as antibody vectors over currently used/tested antibodies: i) the size of these sdAbs is half of the ScFV (~25 kDa) and 10 times smaller than any conventional IgG (150 kDa), ii) they can
5 be expressed in high quantity in the *E. coli* periplasm (Ghahroudi et al., *FEBS Letts.*, **414**, 521-526 (1997)), an important prerequisite for production of recombinant proteins, iii) sdAbs are likely to be cleared faster from the serum and tissues than complete IgG, iv) sdAbs are shown to have a remarkable stability against high temperature, pH, and salts (Muyldermans et al., *supra*),
10 and v) the non-specific interaction of sdAbs with tissues expressing high levels of Fc receptors (e.g., liver, spleen) will be low, since sdAbs lack Fc domain.

15 EXPERIMENTAL

Mouse melanoma, Cloudman S91, clone M-3 cells were obtained from American Type Culture Collection (Rockville, MD). Human lung microvascular endothelial cells (HLMEC) and human umbilical vein
20 endothelial cells (HUVEC) were purchased from Clonetics (San Diego, CA). The fetal human astrocytes (FHAs) were a kind gift from Dr. Jack Antel, MNI, Montreal, Canada.

All culture media and fetal bovine serum (FBS) were obtained from Gibco
25 BRL (Gaithersburg, MD). Endothelial cell growth supplements (ECGS), ITS™ Premix, Matrigel, and human fibronectin were purchased from Collaborative Biomedical Products (Bedford, MA). Human serum, gelatin, bovine serum albumin, and anti-mouse antibody conjugated to alkaline phosphatase or horse radish peroxidase were obtained from Sigma Chemical Co. (St. Louis,
30 MO). Anti-phage V111 coat protein antibody and the Hi-TRAP™ column were purchased from (Amersham Pharmacia Biotech, Montreal, QC, Canada). Precast 4-12 % SDS-PAGE gradient gel and colloidal gold stain were

purchased from Helixx (Ontario, Canada). Silver staining kit was obtained from Bio-Rad (Ontario, Canada).

5 All other biochemical and molecular biology reagents were chemical grade purchased from various companies. Unless stated otherwise, the bacterial media were prepared as described (Sambrook et al., Molecular cloning: A Laboratory Manual (2nd ed.). Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989)). Phosphate-buffered saline (PBS) was prepared as described (Sambrook et al., supra). Induction medium was the same as
10 Terrific Broth except that it contained no salts. Agarose top was prepared by combining the following reagents in a total volume of 1 liter: 10 g bacto-tryptone, 5 g yeast extract, 10 g NaCl, 1 g MgCl₂·6H₂O, and 7 g agarose. The mixture was autoclaved and stored solid at room temperature.

The oligonucleotides were synthesized using the Applied Biosystems 394
15 DNA/RNA synthesizer. DNA sequencing was performed by the dideoxy method (Sanger et al., *Biotechnology*, 104-108 (1992)) using the AmpliTaq DNA Polymerase FS kit and 373A DNA Sequencer Stretch (PE Applied Biosystems, Mississauga, ON, Canada). The host bacteria used for cloning was TG1: *supE hsd5 thi (lac-proAB) F' [traD36 proAB⁺ lac^r lacZ^{M15}]*. All the
20 cloning steps were performed as described (Sambrook et al., supra). The vector fd-tet was purchased from American Type Culture Collection (Manassas, VA) and engineered such that it contained Apal and NotI restriction sites immediately following the gllp leader sequence codons (Simon J. Foote, personal communications).

25

Construction of naive llama sdAb library

Total RNA was isolated from the leukocytes of freshly-drawn heparinized blood of a male Llama (*Lama glama*) using QIAamp RNA Blood MiniTM kit (QIAGEN, Mississauga, ON, Canada) and following the recommended
30 protocol. The concentration of RNA was calculated by measuring the A260 value and assuming 1 A260 = 40 µg/ml. Reverse transcription-polymerase chain reaction (RT-PCR) was performed on a total of 5.3 µg RNA using the HotStarTaq PolymeraseTM kit (QIAGEN). The primers used included a CH2-

specific primer, LlamaFOR, 5'(CGCCATCAAGGTACCAGTTGA)3' [SEQ ID No: 88] and LlamaBACK primer, 5'(GATGTGCAGCTGCAGGCGTCTGGRGGAGG)3' [SEQ ID No: 89], which anneals to the 5' flanking region of VH genes. Amplified product of approximately 600 base pair was purified from the agarose gel using QIAquick Gel ExtractionTM kit (QIAGEN) and subjected to a second round of PCR using the primers LlamaApall, 5'(CATGACCACAGTGCACAGGAKGTSCAGCT)3' [SEQ ID No: 90] and LlamaNotI, 5'(CGATTCTGCGGCCGCTGAGGAGACGGTGACCTG)3' [SEQ ID No: 91]. The PCR mixture contained 10 pmol/ μ l each of the two primers, 1X buffer (Perkin Elmer), 200 μ M each of the four dNTPs and 0.05 unit/ μ l AmpliTaqTM DNA polymerase (Perkin Elmer). PCR protocol consisted of an initial denaturation step at 95°C for 15 min followed by 35 cycles of 94°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The primers were complimentary to the 5' and 3' ends of the amplified product and incorporated Apall and NotI restriction sites (underlined) at the end of VH genes. The amplified products were purified using QIAquick PCR Purification kitTM (QIAGEN), cut sequentially with Apall and NotI restriction endonucleases, purified again, ligated to the Apall/NotI-treated fd-tet phage vector and desalted using the above kit. Electrocompetent TG1 cells were prepared (Tung et al., *Trends Genet.*, 128-129 (1995)) and 1.5 μ g of the ligated product was mixed with 40 μ l of competent E. coli strain TG1 and the cells were transformed by electroporation using the BIO-RAD Gene PulserTM according to the manufacturer's instructions. The transformed cells were immediately transferred into 1 ml of SOC medium and split into 3 sterile tubes containing 3 ml of 50°C agarose top, vortexed immediately, poured onto pre-warmed 2xYT Petri dishes, and incubated at 37°C overnight. The phage particles were eluted by adding five ml of sterile PBS to the plates gently shaken at 4°C for 3 hr. The phage-containing PBS was collected, the plates were rinsed with an additional 5 ml PBS and the two supernatants were combined in a centrifuge bottle. The contents were centrifuged at 6000g for

15 min at 4°C, the supernatant was decanted into a sterile centrifuge bottle and the phage was purified as described (Harrison et al., supra). At the end of the purification, the phage pellet was dissolved in 20 ml of sterile PBS and stored in liquid nitrogen in 100 µl aliquots.

5

To determine the size of the library, immediately following the transformation and after the addition of the SOC medium, a small aliquot of the electroporated cells was serially diluted in exponentially growing TG1 cells. 200 µl of the diluted cells was mixed with 3 ml of 50°C agarose top and immediately poured onto 2xYT plates pre-warmed to 37°C. Plates were incubated overnight at 37°C and the number of plaques was used to determine the size of the library.

Cell Culture

15 Human cerebrovascular endothelial cells (HCEC) and human pial arterial endothelial cells (HPEC) were isolated using a modification (Stanimirovic et al., *J. Cell. Physiol.*, **169**, 455-467 (1996)) of the procedures described by Gerhart et al. (*Brain Res. Bull.*, **21**, 785-793 (1988)). Capillaries and microvessels derived from small samples of human temporal lobe excised surgically from patients treated for idiopathic epilepsy were enzymatically dissociated and used to initiate endothelial cultures in growth media (Earle's salts, 25 mM Hepes, 4.35 g/l sodium bicarbonate and 3 mM L-glutamine), 10% fetal bovine serum (FBS), 5% human serum, 20% murine melanoma cell (mouse melanoma, Cloudman S91, clone M-3, melanin producing cells)-conditioned media, 5 µg/ml of insulin, 5 µg/ml of transferrin, 5 ng/ml selenium, and 10 µg/ml of endothelial cell growth supplement (ECGS), (Stanimirovic et al., supra). The human lung microvascular endothelial cells (HLMEC) and human umbilical vein endothelial cells (HUVEC) were grown and subcultured in the media supplied by the Clonetics (San Diego, CA). The primary fetal human astrocyte cultures (FHAs) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, 2 mM

glutamine, 10% FBS, and 25g/ml gentamycin. All cell cultures were maintained at 37°C in an atmosphere of 5% CO₂ in air.

Differential Panning on Human Endothelial Cells

5 A phage displayed llama single domain antibody library with a functional size of 5.6×10^8 was used for panning. Sub-cultured HLMEC and HCEC (passages 2-6; ~ 1 million cells) were used as bait in each round of panning. The endothelial cells were washed three times in PBS, scraped, counted and blocked by re-suspending the cells in 100µl of PBS containing 1% bovine
10 serum albumin (BSA) solution. Following a 30 min blocking, 10^{12} phage transducing units in 1% BSA-PBS were added to the HLMEC and allowed to bind for 1 h at 37°C. The HLMEC were then centrifuged for 5 minute at 1000 rpm and the supernatant containing the unbound phage was collected and added to the HCEC (100 µl final volume). After 1 h incubation at 37°C, HCEC
15 were washed 6 times with ice cold PBS (200µl) with a short centrifugation (1000 rpm) between the washes. The phage bound to HCEC was stripped from the cell surface in 3 consecutive steps (designated S1, S2, S3) using a 500 µl of stripping buffer [50 mM glycine pH 2.8, 0.5 M NaCl, 2 M urea, and 2% polyvinyl pyrrolidone (PVP)](Becerril et al., *Biochem. Biophys. Res. Commun.*, **255**, 386-393 (1999)). The stripping buffer containing phage
20 dislocated from HCEC was collected and neutralized with half volume of 1M Tris pH 7.4 buffer.

Immediately following 3 stripping rounds, the phage internalized into HCEC
25 (designated INT) was extracted using 200 µl of lysis buffer (100 mM Triethanolamine). Lysis buffer containing internalized phage was collected and neutralized as above.

Approximately 400 µl of S3 and 150 µl of INT phage fractions were
30 subsequently used to infect 10 ml of exponentially growing TG1 culture and allowed to amplify at 37°C for 30 min. Serial dilutions of the infected cells were then used to determine the titre of the phage present in each fraction.

200 µl of the serially diluted cells were mixed with 3 ml of 45°C agarose top and immediately poured onto prewarmed 2xYT plates and incubated overnight at 37°C. The remainder of the infected cells were spun down and resuspended in 900 µl 2xYT, divided in 3 X 300 µl aliquots, mixed with 3 ml
5 agarose top and the phage were plated for overnight propagation at 37°C. The propagated phage particles were then eluted by adding 5 ml of sterile PBS while gently shaking plates at 4°C for 3 h. The phage-containing PBS was then collected; the plates were rinsed with an additional 5 ml PBS and two supernatants were combined in a centrifuge bottle. The contents were
10 centrifuged at 6000g for 15 min at 4°C, the supernatant was decanted into a sterile centrifuge bottle and the phage was purified as described (Harrison et al., *Methods Enzymol.*, **37**, 579-590 1996). The phage titre was determined, and a total of 10^{12} transducing phage units from a S3 fraction of the first panning (1S3) were used for the second round of selection. The third and
15 fourth panning were performed using 10^{12} transducing units of 2S3 and 3S1 phage fractions, respectively.

Plaque PCR

During each round of panning the plaques from the titre plates were randomly
20 chosen for PCR analysis. After a brief vortexing of the plaque in a 50 µl of H₂O, a 1 µl was used as template for PCR amplification using the primers llama Apall, 5'(CATGACCACAGTGCACAGGAKGTSCAGCT)3' [SEQ ID No: 90] and llama NotI, 5'(CGATTCTGCGGCCGCTGAGGAGACGGTGACCTG)3' [SEQ ID No: 91]. The PCR mixture contained 10 pmol/µl each of the two
25 primers, 1X buffer (Perkin Elmer, Mississauga, ON, Canada), 200 µM each of the four dNTP's and 0.05 unit/µl AmpliTaqTM DNA polymerase (Perkin Elmer, Mississauga, ON, Canada). PCR protocol consisted of an initial denaturation step at 95°C for 15 min followed by 35 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 10 min. The
30 amplified products were run on 1% agarose gel; enrichments were assessed by the percent of plaques that yielded a 600 bp full length gene product.

Sequencing

Selected phage clones were PCR amplified using the same set of primers and condition used for colony (plaque) PCR. The amplified products were purified with QIAquick Gel ExtractionTM kit (QIAGEN), and sequenced by the dideoxy method (Sanger *et al.*, 1977) using the AmpliTaq DNA Polymerase FS kit. Analysis was done using 373A DNA Sequencer Stretch (PE Applied Biosystems, Mississauga, ON, Canada).

Construction and purification of c-myc- His₅ -sdAb fusion proteins

Genes of phage-displayed single domain antibodies (sdAb) selected by differential panning and enrichment procedures described above were amplified out of the phage vector by PCR using the primers, VH.Bbs, 5'(TATGAAGACACCAGGCCGATGTGCAGCTGCAGGCG)3', [SEQ ID No: 92] and VH.Bam, 5'(TATGGATCCTGAGGAGACGGTGACCTG)3' [SEQ ID No: 93] that introduced *Bbs*I and *Bam*HI sites at the ends of the amplified fragments. sdAb genes were subsequently purified, cut sequentially with *Bbs*I and *Bam*HI restriction endonucleases, purified again with QIAquick Gel ExtractionTM kit (QIAGEN, Mississauga, ON, Canada), and ligated to the *Bbs*I/*Bam*HI-treated pSJF-2 vector. An aliquot of the ligated product was used to transform *E. coli* strain TG1. Transformants were selected on ampicillin plates and the clones harbouring the sdAb genes were identified by PCR and sequencing. For expression, single positive clones were used to inoculate 25 ml of LB containing 100 µg/ml ampicillin and the culture was shaken at 240 rpm at 37°C overnight. The entire overnight culture was used to inoculate 1 liter of M9 medium supplemented with 5 µg/ml vitamin B1, 0.4% casamino acid and 100 µg/ml ampicillin. The culture was shaken at room temperature for 30 h at 180 rpm and subsequently supplemented with 100 ml of 10X induction medium and 100 µl of 1 M isopropylthio-D-galactoside (IPTG). The culture was shaken for another 60 h and the periplasmic fraction was extracted by osmotic shock method (Anand *et al.*, *Gene*, 100, 39-44 (1991)). The periplasmic fraction was dialyzed extensively in 10 mM HEPES buffer pH 7.0, 500 mM NaCl.

The presence of the C-terminal His₅ tag in sdAbs allowed for one step protein purification by immobilized metal affinity chromatography using HiTrap ChelatingTM column. The 5-ml column was charged with Ni²⁺ by applying 30 ml of a 5 mg/ml NiCl₂.6H₂O solution and subsequently washed with 15 ml deionized water. Purification was carried out as described (MacKenzie et al., *Biotechnology*, 12, 390-395 (1994)) except that the starting buffer was 10 mM HEPES buffer, 10 mM imidazole, 500 mM NaCl, pH 7.0. The bound protein was eluted with a 10-500 mM imidazole gradient. The purity of the protein was determined by SDS-PAGE (Laemmli U.K., *Nature*, 227, 680-685 (1970)).

Binding and uptake of sdAb-c-myc-His₅ fusion proteins

The sdAbs were labeled with alexafluor 488/532 (Molecular Probes, Ontario, Canada) and purified on a gel filtration column as per the manufacturer protocol.

The plasma membrane fraction of the HCEC was prepared using a detergent free density gradient medium OptiPrepTM (Nycomed Pharma AS, Norway). The membrane vesicles were prepared by sonication (50J/W per sec each time) and purified as described (Smart et al., *Proc. Natl. Acad. Sci. USA*, 92, 10104-10108 (1995)). The membranes and vesicles were applied onto a coverslip and air dried before the addition of Alexafluor 488/532-labeled sdAb. After 1 h of incubation, the coverslips were rinsed with PBS and imaged using an Olympus fluorescence microscope.

25

To determine the uptake of FC44, FC5 and NC11 into live cells, HCEC and HLMEC were plated onto glass coverslips in 24 well dishes. Cells were incubated with the Alexafluor 488/532-labeled sdAbs for up to 3 h in PBS at 37°C and in some cases at 22°C and 4°C. Cells were then washed 2 times in PBS and examined using confocal/fluorescence microscopy. The viability of

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cell subjected to the described treatments was assessed by propidium iodide staining of cell nuclei (Stanimirovic et al., *Glia*, 19, 123-134 (1997)).

Transmigration studies in *in vitro* BBB model

- 5 HCEC were seeded at 3×10^5 cells/cm² on a 0.5% gelatin coated Falcon tissue culture inserts (pore size-1 μ m; surface area 0.83 cm²) in 1 ml of growth medium. The bottom chamber of the insert assembly contained 2 ml of growth medium supplemented with the fetal human astrocyte (FHAs)-conditioned medium in a 1:1 (v/v) ratio (Muruganandam et al., *supra*). The
- 10 FHAs-conditioned medium was obtained by incubating confluent FHAs in a serum free M199 for 72 h. The transendothelial electrical resistance (TEER) of the endothelial cell monolayers was measured using Endohm™ electrical resistance apparatus (World Precision Instruments, Sarasota, FL).
- 15 All transport experiments were performed in transport buffer (HBSS containing 5 mM glucose/10 mM HEPES pH 7.4/ 0.05% BSA) after equilibrating the cells for 30 min at 37°C. The barrier integrity was assessed by measuring the passage sodium fluorescein (MW 376 Dalton 25 μ g/ml), fluorescently-labeled dextran (MW 10 kDa), and ¹⁴C dextran-carboxyl (MW
- 20 70 kDa) across triplicate HCEC monolayers, and across 0.5% gelatin coated inserts without cells. Samples were collected from the bottom chambers from 5 min to 90 min period. The fluorescence was measured in a Cytofluor 2350, Millipore, Ont, Canada and the radioactive counts were measured in Wallac microbeta liquid scintillation counter (Turku, Finland). Clearance values were
- 25 determined using previously described protocols (Dehouck et al., *J. Neurochem.*, 58, 1790-1797 (1992); Pardridge et al., *J. Pharmacol. Exp. Therap.*, 253, 884-891 (1990)).

- 30 The selection of BBB-transmigrating phage clones was done by adding amplified library containing 10^{11} transducing phage enriched for HCEC binding (4S3) and internalizing species (4INT) to the upper compartment

and by determining the PFU's in 10µl out of 25µl aliquots collected from the bottom compartment at specified time points.

5 The ability of purified soluble FC44, FC5, and NC11 (negative control) fusion proteins to transigrate across *in vitro* human BBB model was assessed by adding 100 µg of fusion protein into the upper compartment. At selected time points, 200 µl of the sample from the bottom compartment was collected and the concentration of the respective sdAb in the bottom chamber was quantified by a Nickel-trap ELISA as described below.

10

Enzyme-linked immunosorbent assays (ELISA)

ELISA against phage coat protein P8 (phage ELISA) was used to determine the binding of selected phage clones to various human endothelial cells in culture. Individual phage-infected TG1 colonies were used to inoculate 200 µl of Luria broth (LB) in sterile 96-well plates. The cells were grown overnight gently shaken at 100 rpm at 37°C. The plates were then spun down, and the phage-containing supernatants were used for phage ELISA as described below. Briefly, monolayers of HCEC and HLMEC grown in 96 well plates were blocked at room temperature by adding 300 µl of PBS-2% BSA for 1 h.

15 The contents of the wells were aspirated, 100 µl of phage supernatant in 2% BSA-PBS was added, and cells were incubated at 37°C for 1 h. After removing the supernatants, the cells were washed 6 times with PBS-0.05% (v/v) Tween-20 and then incubated with 100 µl of a 1:1000 dilution of HRP-conjugated anti-M13 monoclonal antibody in 2% BSA-PBS at 37°C for 1 h.

20 The cells were washed 6 times and the binding of phage to the cell surface was detected colorimetrically by adding 100 µl of the TMB peroxidase substrate and H₂O₂ (KPL, Maryland, USA) mixture at room temperature. The reaction was terminated by adding 100 µl of 1 M H₃PO₄ and the optical density (O.D) was read at 450 nm using DYNATECH MR5000 ELISA plate reader (Dynatech Laboratories, Chantilly, VA, USA). Wild type phage was used for determining a non-specific binding.

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ELISA against c-myc tag was used to determine binding of purified sdAb-c-myc-His₅ fusion proteins to cells or cell lysates. The recombinant sdAb (100µg/ml) fused with the c-myc tag were incubated with the live HCEC and HLMEC grown in 96 well-plates, or with the cell extracts from HCEC, HPEC, FHAs, HUVEC and HLMEC for 1h at 37°C. After washing to remove unbound sdAb-c-myc fusion protein, the bound protein was detected using mouse anti-c-myc antibody conjugated to HRP (1: 5000 dilution).

A nickel trap ELISA was used to determine the concentration of purified FC5, FC44, and NC11 fusion proteins in the BBB transmigration assay. Briefly, the sample aliquots from the bottom chambers of the *in vitro* BBB model were allowed to incubate overnight in a 96 format wells of Ni-NTA HisSorb strips (Qiagen Inc, Mississauga, ON, Canada) with constant shaking. After blocking the wells with 2% BSA-PBS, the concentration of His₅-sdAbs trapped onto the Ni-NTA HisSorb was detected using an HRP-conjugated anti-c-myc antibody (1:5000 dilution). The concentration was determined from standard curve constructed using respective pure sdAbs.

***In vivo* biodistribution of the FC5 and FC44**

Five to six week old C57BL/6 mice (approx. 18 g) were injected in the tail vein with phage-displayed FC5, FC44 or wild type phage (10⁹ pfu/mouse in 100 µl for each library). Separate groups of animals were injected in the tail vein with 30 µg/mouse of FC5, FC44 or NC11 fusion proteins with c-myc-His₅. Four hours after injection, the animals were anesthetized with 0.27 ml/100g of Hypnorm/Versad i.p. and perfused through the heart with 50 ml of saline. Brain, liver, lung and kidney were dissected, snap-frozen in dry ice and stored at -80°C.

Phage titers and immunoblot analyses in these tissues were done after homogenizing a pre-weighed tissues in 0.5 ml PBS containing 10µg/ ml of protease inhibitors using FastPrep[®] FP 120 instrument. For determining phage titers, known amounts of tissue extract (0.2 to 10 µl) were used for

infecting the bacteria and titers were determined as described above. For immunoblot, tissue was homogenized and 500 µg of the protein from the tissue extracts was affinity purified using His Microspin™ purification module. After desalting the final eluate, samples were dried in a vacuum concentrator, resuspended in 80 µl of SDS-PAGE sample buffer, and run on 10% SDS-PAGE.

Affinity purification of the antigen

HCEC grown in 100 mm tissue culture dishes were rinsed and scraped into PBS cooled to 4°C. The cells were briefly sonicated in a bucket sonicator for 3 min in the presence of 0.05% Triton-X 100 and 10 µg/ml protease inhibitors and, after determining the protein content in the cell lysates by the method of Bradford (*Anal. Biochem*, 72, 248-254 (1976)) the antigen was affinity purified using His Microspin™ purification module (Amersham Pharmacia Biotech, Quebec, Canada). A known amount of protein was loaded on to a nickel-charged chelating sepharose microspin column previously adsorbed with His₆ tagged fusion sdAb. Sequential washing and elution was done using increasing strengths (20 mM, 100 mM and 400 mM) of the imidazole/salt elution buffer.

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Immunoblot

Immunoblot analysis was used to determine the presence of sdAb fusion proteins in tissue extracts after *in vivo* injection, and the expression of the antigen in HCEC. The affinity purified tissue extracts and antigen-sdAbs complexes were mixed with an equal volume of gel loading buffer (50 mM Tris-HCl pH 6.8, 100 mM DDT, 2% SDS, 0.1% bromophenol blue, 10% glycerol), boiled for 3 min and loaded onto a 4-12% or 12 % SDS polyacrylamide gels. The proteins separated on the gel were subsequently electrophoretically transferred onto a nitrocellulose membrane using a Bio-Rad mini transblot cell apparatus. The membranes were blocked with PBS-BSA for 1 h.

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For sdAbs detection in mouse tissues, the membranes were incubated with monoclonal anti-c-myc IgG antibody (1:1000) for 30 min, washed and then incubated with secondary anti-mouse IgG antibody conjugated to alkaline phosphatase for 30 min.

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For HCEC antigen detection, a separate gel was silver stained using the Silver plus kit (BioRad). Nitrocellulose membranes were incubated with phage carrying selected sdAbs (10^9 pfu) or with 30 μ g of purified selected sdAbs for 1 h at room temperature under constant agitation. After washing (3 x 5 min) in PBS containing 0.05% Tween-20, the membranes were incubated with monoclonal anti-c-myc IgG antibody (1:1000) for 0.5 h or with anti-MI3 phage antibody. The membranes were then washed 3 X 5 min in PBS and incubated with secondary anti-mouse IgG antibody conjugated to alkaline phosphatase for 0.5 hour.

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The protein bands were visualized using the alkaline phosphatase substrates, 5-bromo-4-chloro-3 indolyl phosphate and nitroblue tetrazolium (Sigma Chemical Company, St.Louis MO).

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Although various particular embodiments of the present invention have been described hereinbefore for the purpose of illustration, it would be apparent to those skilled in the art that numerous variations may be made thereto without departing from the spirit and scope of the invention, as defined in the appended claims.

25

WHAT IS CLAIMED IS:

1. A process for enriching a phage display library of antigen-binding antibody fragments derived from llama antibodies with phage species binding selectively to human endothelial cells expressing BBB antigens,
5 each antigen-binding fragment comprising at least a part of the variable heavy domain (V_{HH} or VH) of a llama antibody, said process comprising the steps of:
 - (a) adsorbing the phage display library on first endothelial cells which do not express BBB antigens,
 - 10 (b) adsorbing phage species unbound to the first endothelial cells on second endothelial cells which express BBB antigens, and
 - (c) stripping and recovering phage species bound to the second endothelial cells.
- 15 2. A process according to claim 1, which process further comprises the step of lysing the second endothelial cells following step (c) and recovering phage species internalized in the second endothelial cells.
- 20 3. A process according to claim 1, wherein the stripping is repeated at increasing levels of stringency and only phage species stripped at the highest level of stringency are recovered.
4. A process according to claim 3, wherein steps (a), (b) and (c) are repeated at least once for the phage species recovered in step (c).
- 25 5. A process according to claim 4, wherein prior to repeating steps (a), (b) and (c) the recovered phage species are amplified.
6. A process according to claim 2, wherein steps (a), (b), and (c) are
30 repeated at least once for the recovered internalized phage species.
7. A process according to claim 6, wherein prior to repeating steps (a), (b) and (c) the recovered internalized phage species are amplified.

8. A process according to claim 1, wherein the first endothelial cells are human lung microvascular endothelial cells (HLMEC).
- 5 9. A process according to claim 1, wherein the second endothelial cells are human cerebromicrovascular endothelial cells (HCEC).
- 10 10. A process according to claim 1, wherein the antigen-binding antibody fragment derived from a llama antibody comprises a complete variable heavy domain (V_{HH} or VH) of the llama antibody.
- 15 11. A process according to claim 10, wherein the antigen-binding antibody fragment consists essentially of the variable heavy domain of a llama antibody.
- 20 12. A process according to claim 11, wherein the phage display library is derived from the antibody repertoire of a non-immunized llama.
13. A process according to claim 13, wherein the size of the library is of at least 10^8 .
- 25 14. A process according to claim 13, wherein the phage is a modified fd-tet phage.
15. A process for the preparation of a human blood-brain barrier (BBB) transmigrating antibody fragment derived from a llama antibody, said process comprising the steps of:
- 30 (a) providing a phage display library of antigen-binding antibody fragments derived from llama antibodies,
- (b) enriching the library for phage species selectively binding to endothelial cells expressing BBB antigens,

- (c) recovering from the enriched library phage particles selective to the endothelial cells expressing BBB antigens by comparing their binding to various endothelial cells from peripheral and brain tissues,
- (d) enriching the library for phage species internalized into the endothelial cells expressing BBB antigens,
- (e) identifying in the enriched libraries from steps (b) and (d) phage particles transmigrating across an *in vitro* BBB and recovering such phage particles,
- (f) comparing nucleotide sequences of DNA of phage particles recovered in steps (c) and (e) and selecting particles having both high selectivity to the endothelial cells expressing BBB antigens and transmigrating across the *in vitro* BBB,
- (g) amplifying DNA of the phage particles identified in step (f) and cloning into an expression vector a piece of DNA coding for the antibody fragments displayed by the phage particles,
- (h) transforming host cells with the expression vector,
- (i) incubating the transformed cells under conditions allowing the expression of the cloned piece of DNA, and
- (j) recovering the antigen-binding antibody fragment so prepared.
16. A process according to claim 15, wherein the endothelial cells are human cerebromicrovascular endothelial cells (HCEC).
17. A process according to claim 16, wherein the cells internalize the transmigrating antibody fragment.
18. A process according to claim 17, wherein the human brain-blood barrier (BBB) transmigrating antibody fragment derived from a llama antibody comprises at least a part of the variable heavy domain ($V_{\text{H}}\text{H}$ or VH) of the llama antibody.

19. A process according to claim 18, wherein the transmigrating antibody fragment comprises a complete variable heavy domain (V_{HH} or V_H) of the llama antibody.
- 5 20. A process according to claim 19, wherein the transmigrating fragment consists essentially of the variable heavy domain of the llama antibody.
21. A process according to claim 20, wherein the transmigrating antibody fragment is selected from the group consisting of polypeptides designated
10 as FC5 [SEQ ID No. 85], FC 44 [SEQ ID No. 86] and FC 7 [SEQ ID No. 87].
22. An antibody fragment derived from a llama antibody, said fragment capable of transmigrating across the human blood-brain barrier (BBB) and
15 selectively binding to an antigen on the surface of mammalian cells expressing BBB antigens.
23. An antibody fragment according to claim 22, wherein the mammalian cells are human cerebromicrovascular endothelial cells (HCEC).
20
24. An antibody fragment according to claim 23, wherein the antibody fragment is internalized by the HCEC.
25. An antibody fragment according to claim 24, wherein the antibody
25 fragment comprises at least a part of the variable heavy domain (V_{HH} or V_H) of the llama antibody.
26. An antibody fragment according to claim 25, wherein the antibody
30 fragment comprises a complete variable heavy domain (V_{HH} or V_H) of the llama antibody.

27. An antibody fragment according to claim 26, wherein the antibody fragment consists essentially of the variable heavy domain (V_HH or VH) of the llama antibody.

5 28. An antibody fragment according to claim 27, wherein the antibody fragment is selected from the group consisting of polypeptides designated as FC5 [SEQ ID No. 85], FC 44 [SEQ ID No. 86] and FC 7 [SEQ ID No. 87].

10 28. An antibody fragment according to claim 27, wherein complementarity determining regions (CDR) of the fragment are essentially free of cysteine residues.

15 29. An antibody fragment according to claim 27, wherein the CDR1/H1 region of the variable heavy domain (V_HH or VH) is selected from the group consisting of :

VRTFSIYAMG (SEQ ID No: 94)

GFKITHYTMG (SEQ ID No: 95)

VRTFSIYAIG (SEQ ID No: 96).

20

30. An antibody fragment according to claim 27, wherein the CDR2 region of the variable heavy domain (V_HH or VH) is selected from the group consisting of :

GINRSGDVTKYADFVKG (SEQ ID No: 97)

25 RITWGGDNTFYNSVK (SEQ ID No: 98).

31. An antibody fragment according to claim 27, wherein the CDR3 region of the variable heavy domain (V_HH or VH) is selected from the group consisting of:

30 TWAYDTVGALTSGYNF (SEQ ID No: 99)

GSTSTATPLRVDY (SEQ ID No: 100).

32. An antibody fragment according to claim 27, wherein amino acid residues of the VL interface of the variable heavy domain (V_HH or VH) are Glu at position 44, Arg at position 45 and Phe at position 47.

5 33. A therapeutic or diagnostic agent, said agent comprising an antibody fragment derived from a llama antibody, said fragment capable of selectively binding to an antigen on the surface of mammalian cells expressing BBB antigens and transmigrating across the human blood-
10 brain barrier (BBB), said antibody fragment being linked, directly or indirectly, covalently or non-covalently, to a therapeutic or diagnostic entity.

15 34. A therapeutic or diagnostic agent according to claim 33, wherein the mammalian cells are human cerebromicrovascular endothelial cells (HCEC).

35. A therapeutic or diagnostic agent according to claim 34, wherein the antibody fragment is internalized by the HCEC.

20 36. A therapeutic or diagnostic agent according to claim 35, wherein the agent is administered by injection.

25 37. A therapeutic or diagnostic agent according to claim 36, wherein the agent is administered by an intravenous, intraperitoneal, intramuscular, or subcutaneous injection.

30 38. A therapeutic or diagnostic agent according to claim 36, wherein the agent is localized in brain parenchyma after being administered by injection.

39. A therapeutic or diagnostic agent according to claim 35, wherein the antibody fragment comprises at least a part of the variable heavy domain (V_HH or VH) of the llama antibody.

40. A therapeutic or diagnostic agent according to claim 39, wherein the antibody fragment comprises a complete variable heavy domain (V_{H} H or VH) of the llama antibody.
- 5 41. A therapeutic or diagnostic agent according to claim 40, wherein the antibody fragment consists essentially of the variable heavy domain (V_{H} H or VH) of the llama antibody.
- 10 42. A therapeutic or diagnostic agent according to claim 41, wherein the antibody fragment is selected from the group consisting of polypeptides designated as FC5 [SEQ ID No. 85], FC 44 [SEQ ID No. 86] and FC 7 [SEQ ID No. 87]
- 15 43. A therapeutic or diagnostic agent according to claim 34, wherein the therapeutic or diagnostic entity does not permeate the BBB,
44. A therapeutic or diagnostic agent according to claim 43, wherein the therapeutic or diagnostic entity is a peptide.
- 20 45. A therapeutic or diagnostic agent according to claim 45, wherein the peptide is c-myc or His₅ tag.
44. A therapeutic or diagnostic agent according to claim 43, wherein the therapeutic or diagnostic entity is a piece of DNA.
- 25 45. A therapeutic or diagnostic agent according to claim 43, wherein the therapeutic or diagnostic entity is a phage.
- 30 46. A therapeutic or diagnostic agent according to claim 43, wherein the therapeutic or diagnostic entity is a fluorescent or radioactive label.

47. A therapeutic or diagnostic agent according to claim 43, wherein the antibody fragments are linked to a liposomal or polymeric preparation comprising the therapeutic or diagnostic entity.

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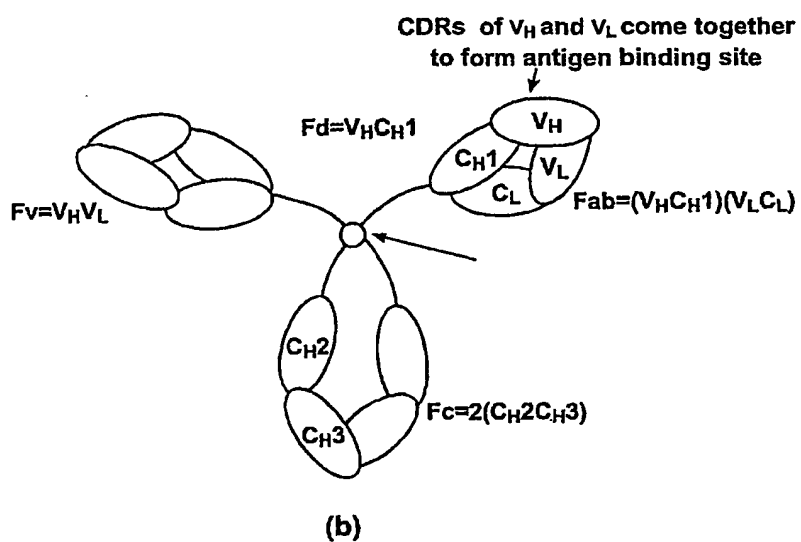
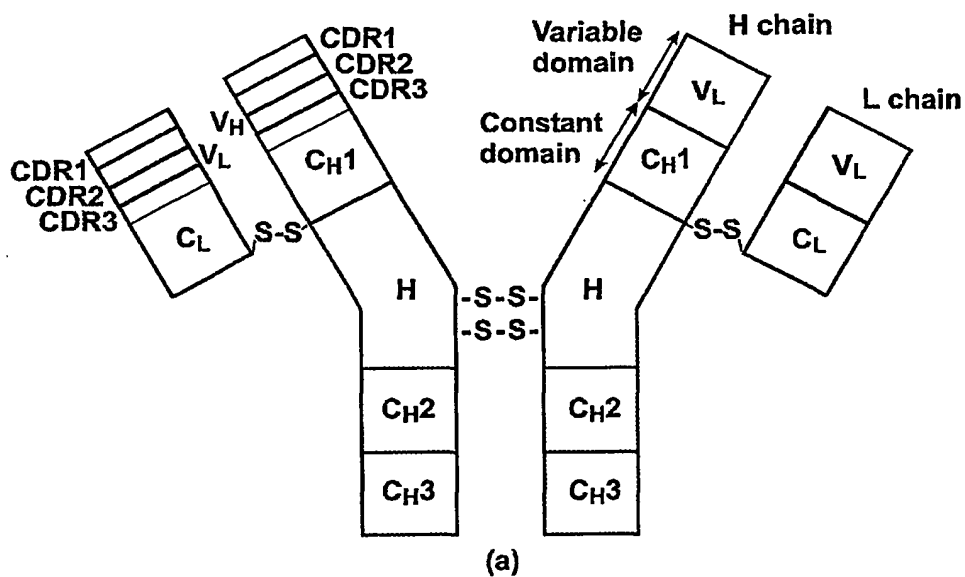


FIG. 1

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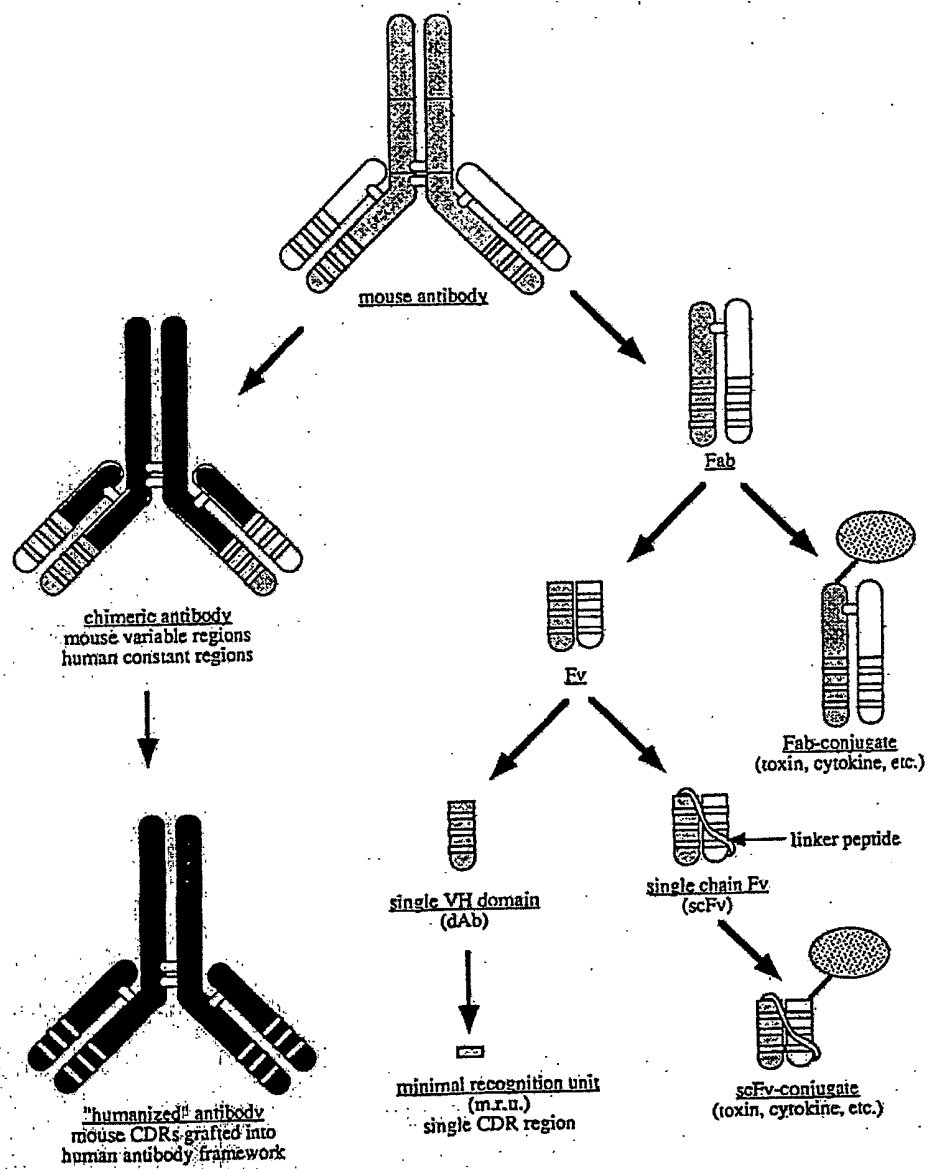


FIG. 2

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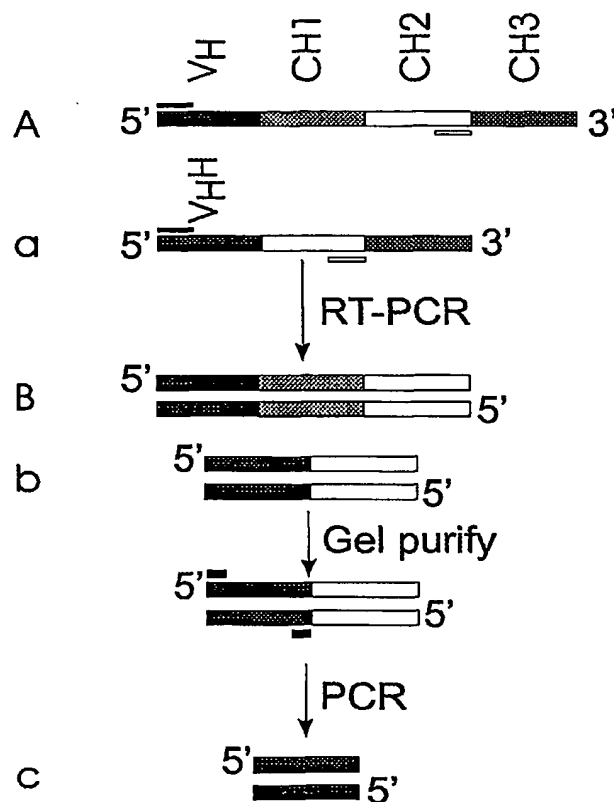


FIG. 3

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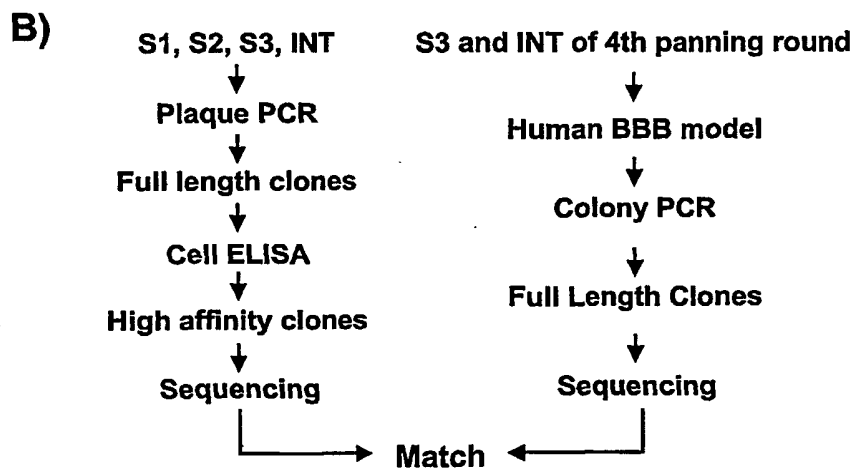
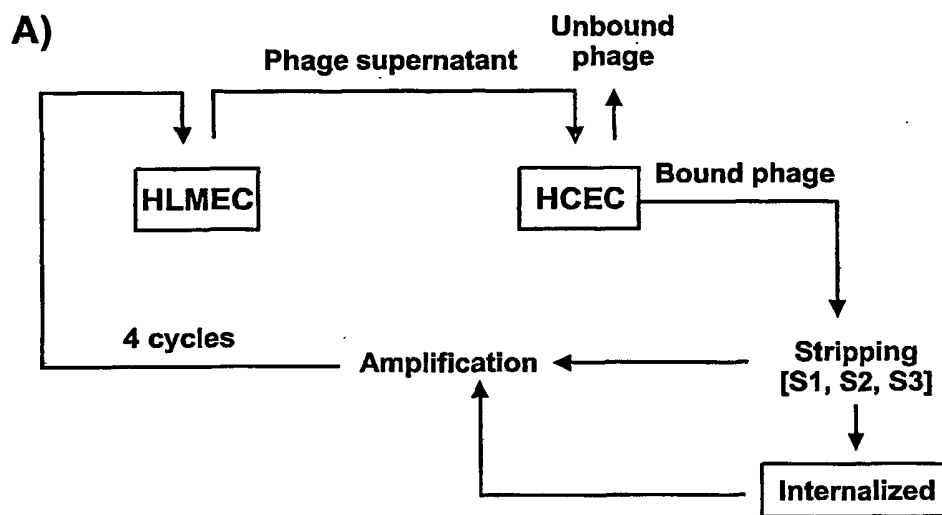


FIG. 4

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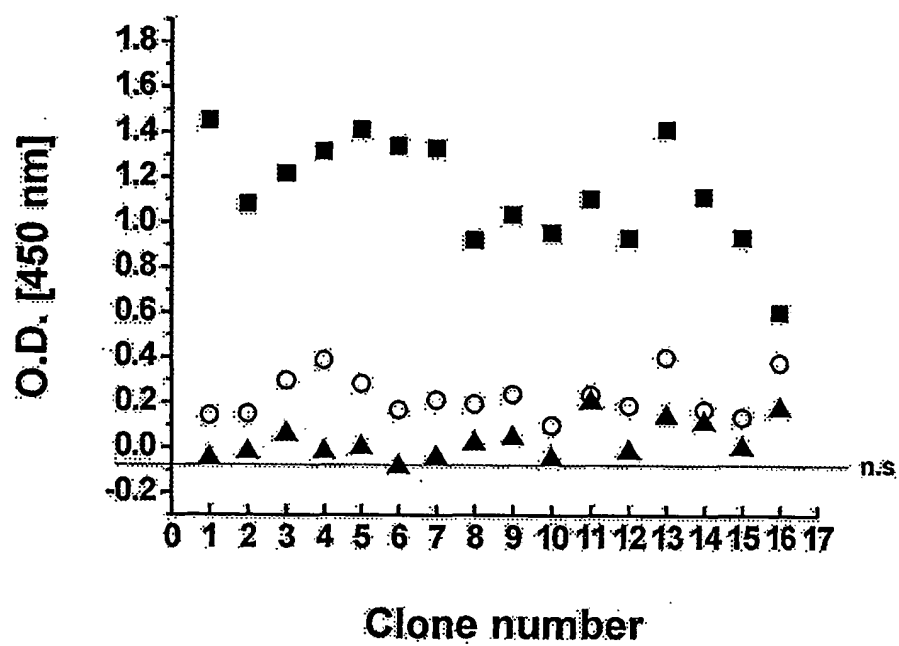


FIG. 5

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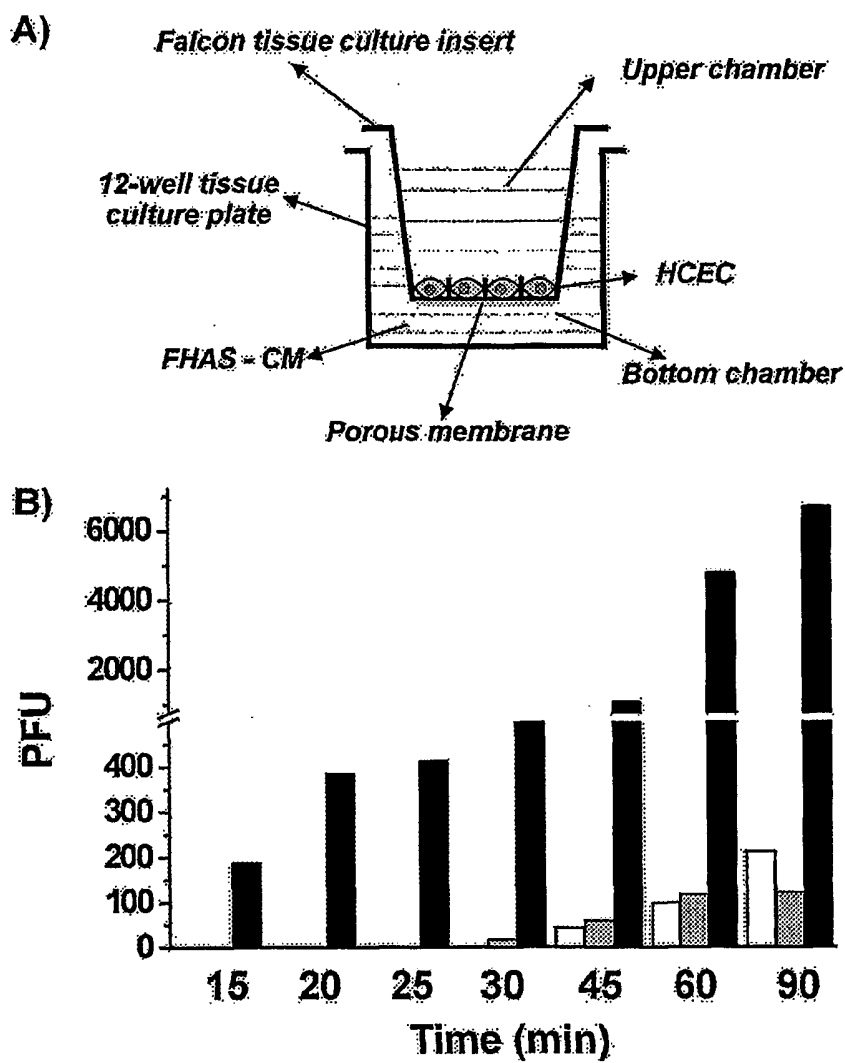


FIG. 6

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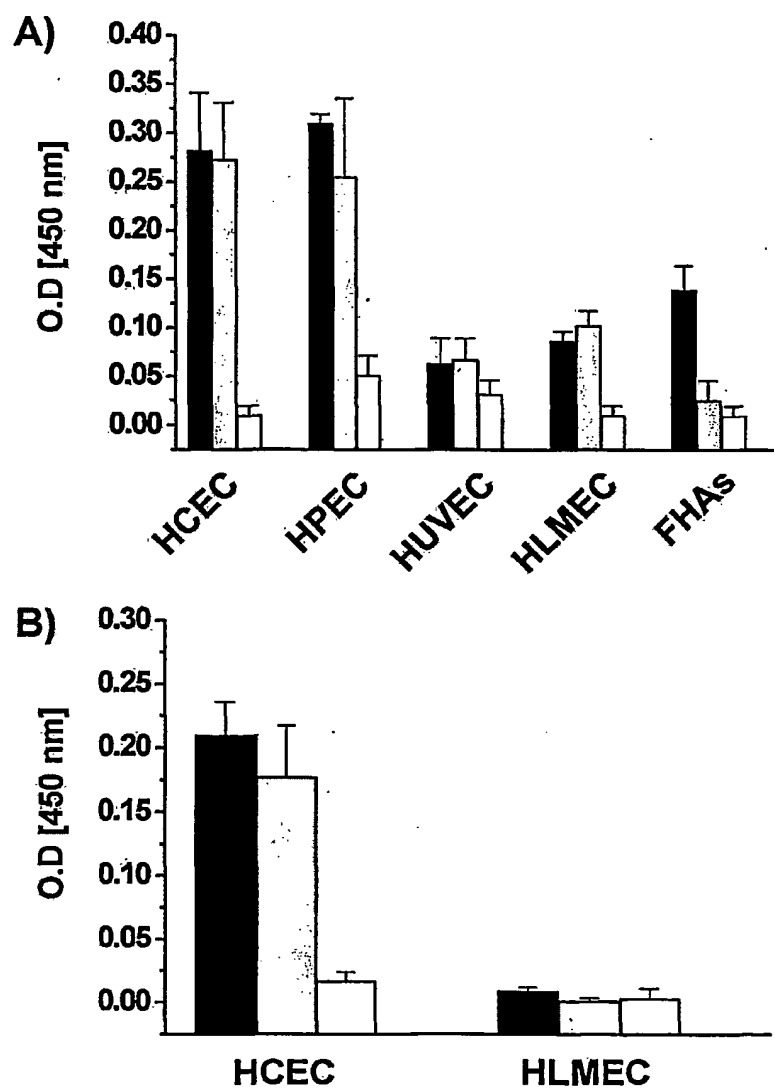


FIG. 7

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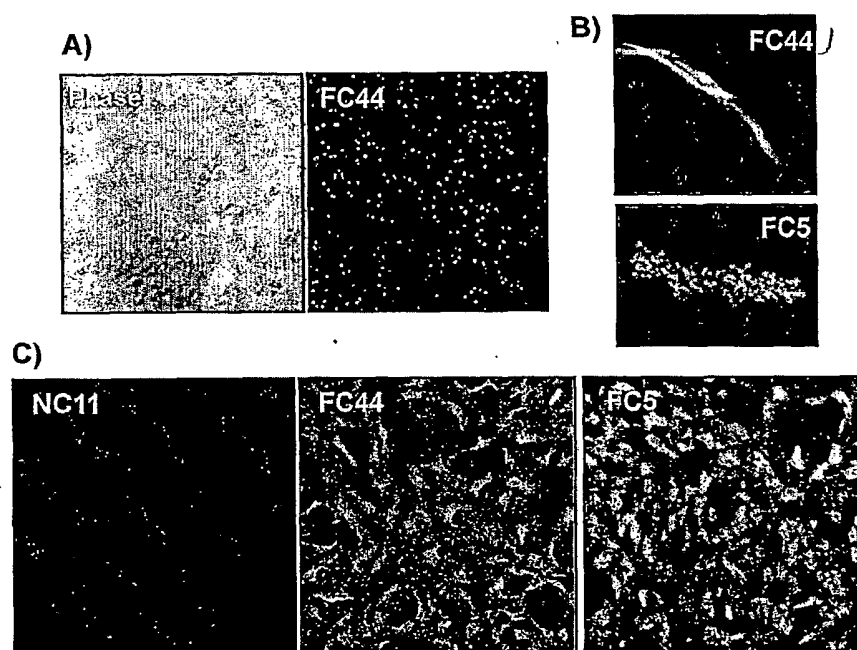


FIG. 8

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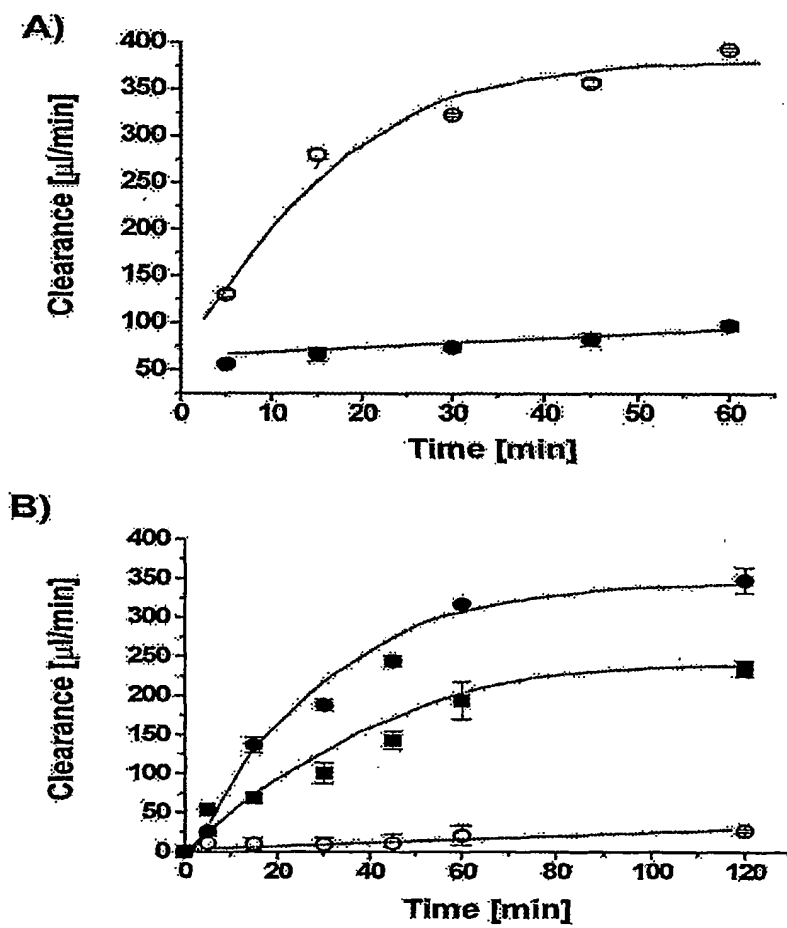


FIG. 9

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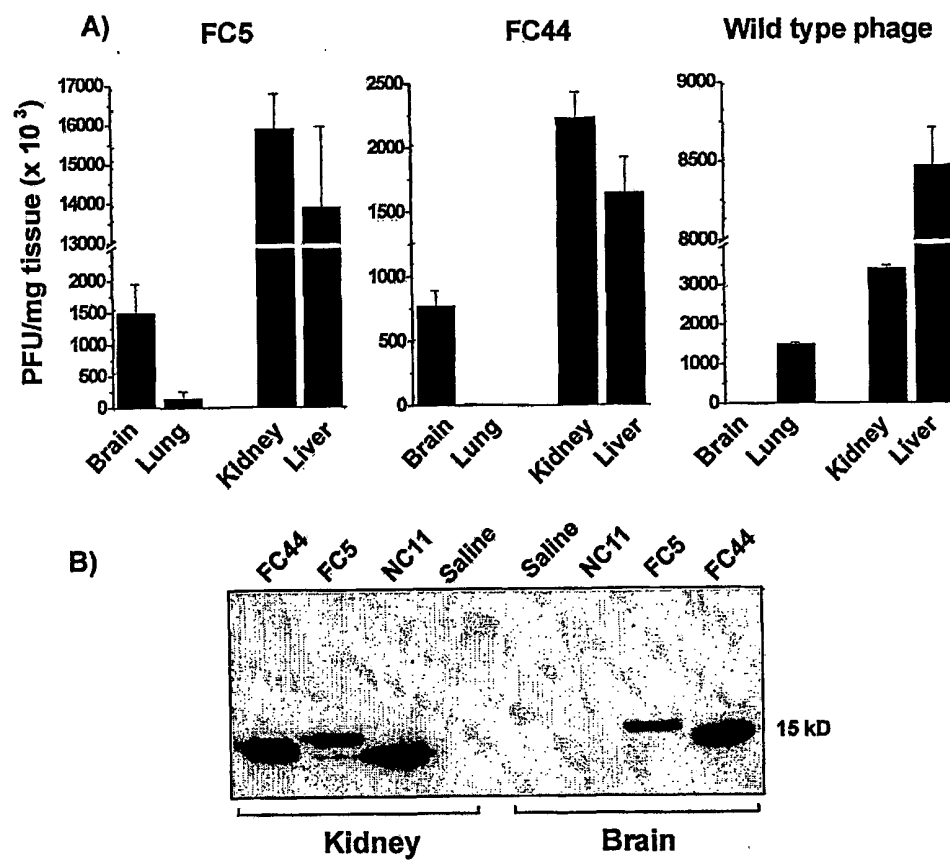


FIG. 10

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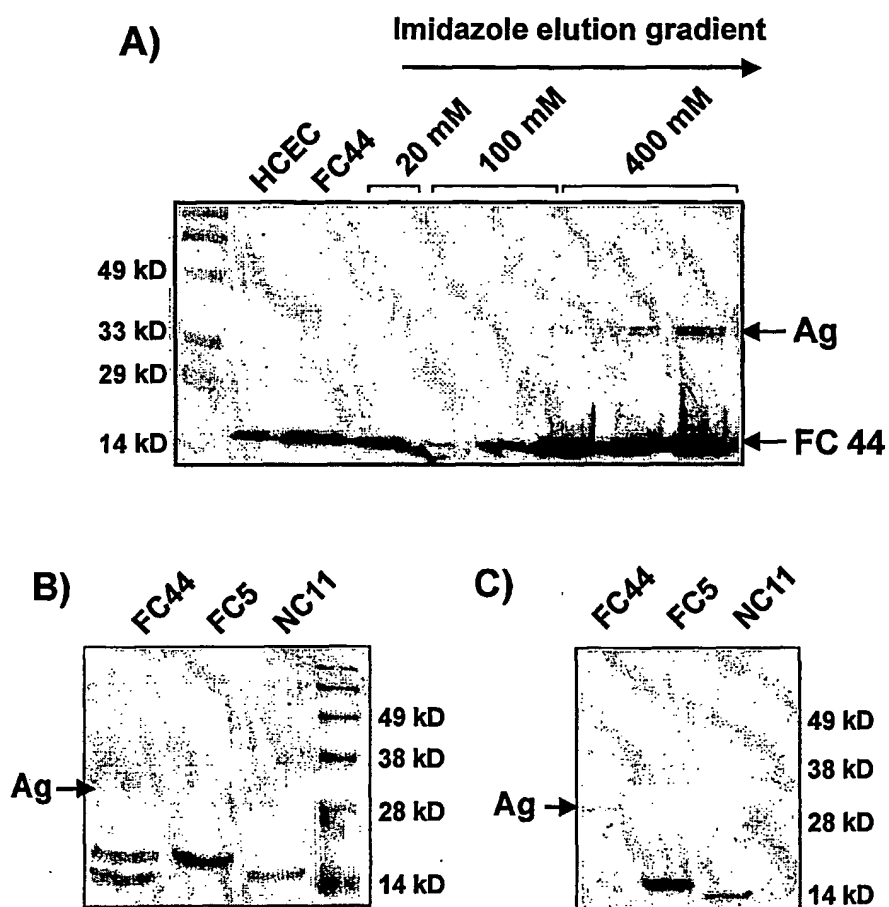


FIG. 11

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA 01/00783

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/10 C07K16/00 C07K16/28 A61K39/395 G01N33/577
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, CHEM ABS Data, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	S. MORRISON ET AL.: "Genetically engineered antibodies and their application to brain delivery." ADVANCED DRUG DELIVERY REVIEWS, vol. 15, no. 1-3, 1995, pages 147-175, XP001031375 page 150, left-hand column, line 16 -page 152, left-hand column, line 13 page 160, right-hand column, line 4 -page 166, left-hand column, line 6 figure 3C	1-47
Y	WO 00 43507 A (UNILEVER PLC ET AL.) 27 July 2000 (2000-07-27) examples claims --- -/--	1-47

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 04678 A (VRIJE UNIVERSITEIT BRUSSEL ET AL.) 3 March 1994 (1994-03-03) claims 24-27 -----	1-47
A	N. STERNBERGER ET AL.: "Blood-brain barrier protein recognized by monoclonal antibody." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE U.S.A., vol. 84, no. 22, November 1987 (1987-11), pages 8169-8173, XP002188811 Washington, DC, USA the whole document -----	1-47
A	J. LI ET AL.: "Genetically engineered brain drug delivery vectors: cloning, expression and in vivo application of an anti-transferrin receptor single-chain antibody-streptavidin fusion gene and protein." PROTEIN ENGINEERING, vol. 12, no. 9, September 1999 (1999-09), pages 787-796, XP000984720 Oxford, GB abstract -----	1-47
A	US 5 855 885 A (CHISWELL ET AL.) 5 January 1999 (1999-01-05) example 4.1 -----	45

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 01/00783

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
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WO 9404678	A	03-03-1994	EP 0584421 A1 AT 203767 T AU 701578 B2 AU 4949793 A AU 9813198 A CA 2142331 A1 DE 69330523 D1 DK 656946 T3 WO 9404678 A1 EP 1087013 A2 EP 0656946 A1 FI 950782 A JP 8500487 T US 5874541 A US 5800988 A US 6015695 A US 5840526 A US 6005079 A US 5759808 A ZA 9306086 A	02-03-1994 15-08-2001 04-02-1999 15-03-1994 25-03-1999 03-03-1994 06-09-2001 05-11-2001 03-03-1994 28-03-2001 14-06-1995 20-04-1995 23-01-1996 23-02-1999 01-09-1998 18-01-2000 24-11-1998 21-12-1999 02-06-1998 22-07-1994
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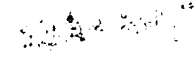
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